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**The role of GRASP65 and GRASP55 in C-terminal valine-dependent  
transport to the cell surface of transmembrane proteins**

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# CHAPTER I

## 1. An overview of the secretory pathway

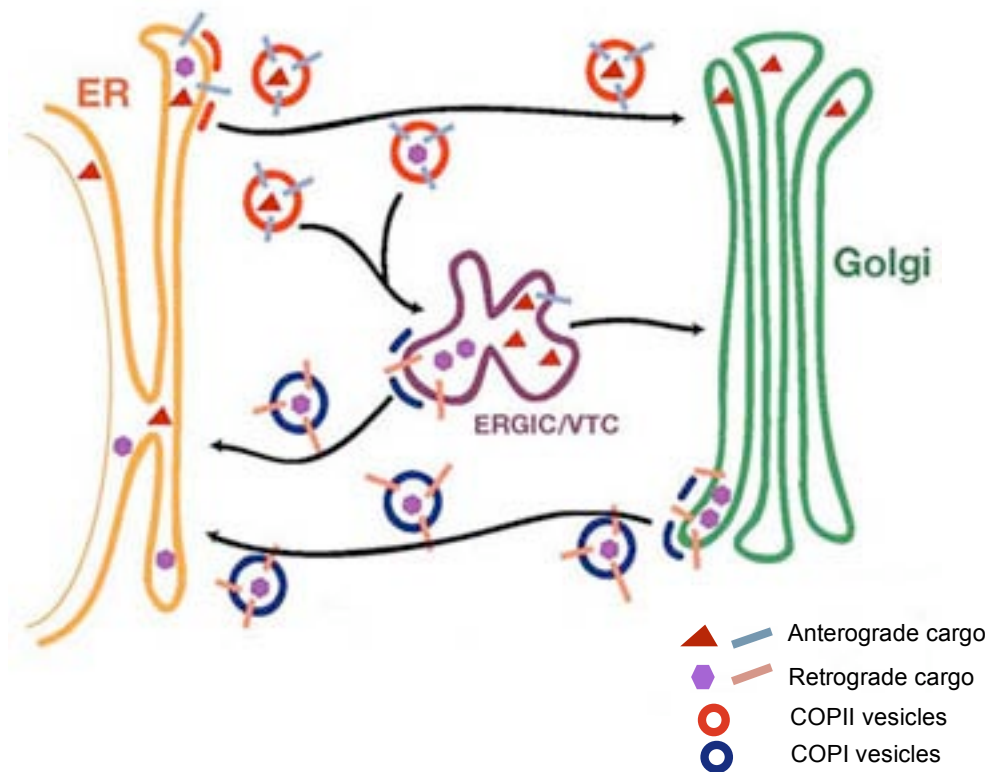
### *1.1 The secretory pathway*

One third of the proteins encoded by the eukaryotic genome are synthesized on ER-bound ribosomes and are destined to be secreted or to localize in one of the organelles of the secretory pathway. In the endoplasmic reticulum (ER), which is also the major site of cellular sterol and lipid synthesis, neosynthesized products begin their journey through the secretory pathway to reach their final destinations. The secretory membrane system is primarily responsible for their distribution to the endomembrane compartments and their delivery to the exterior of the cells. The secretory membrane system is made up of distinct organelles including the ER, the Golgi complex, endosomes, lysosomes and plasma membrane. Within this system, soluble and membrane-bound cargo flows in a highly organized and directional way.

Cargo molecules are first transported from the ER to the Golgi apparatus by the mobile elements of the intermediate compartment after which then pass through the Golgi stacks via cisternal maturation and vesicular/tubular traffic.

In the Golgi apparatus most proteins and many lipids undergo post-translational modifications including glycosylation, proteolytic processing, sulfation and phosphorylation. The Golgi apparatus is also considered the central station for the sorting of cargo molecules. At the exit face of the organelle, the Trans Golgi Network, the lipids and proteins are sorted to pleiomorphic carriers for further transport to the plasma membrane and the

endosome/lysosome system. In order to maintain the organelles' homeostasis this directional membrane flow is balanced by retrieval pathways that bring membranes and selected proteins back to their compartment of origin (Fig.1).



(Modified drawing from Schekman lab website)

**Figure 1. Schematic representation of the secretory pathway.** After their correct folding newly synthesized proteins leave the ER via COPII vesicles. COPI coatamer vesicles mediate retrograde transport from the Golgi complex to the ER.

## 1.2 The endoplasmic reticulum

The point of entry for proteins destined for the secretory pathway is the ER. These proteins are synthesized by ER-associated ribosomes and co-

translationally translocated across the membrane through the Sec61 complex. They start to fold co-translationally. Within the lumen of the ER, newly synthesized proteins find the most favourable environment for their maturation: high calcium concentration, the right redox potential and a set of chaperones that assist the folding process as well as enzymes that operate several co- and post-translational modifications such as signal sequence cleavage, N-linked glycosylation, disulphide-bond formation, and the addition of glycosylphosphatidylinositol anchors. It is here that individual folded subunits before assembly and oligomerization take place.

Aberrant proteins which can originate from mutations or unbalanced subunit synthesis are extremely harmful to cells. Stringent quality-control systems ensure that only correctly folded proteins are sent to their final destinations and address the misfolded proteins to their degradation. A proper balance between synthesis, maturation and degradation is crucial for cell survival. In some cases, for a number of reasons, this sophisticated system can fail and lead to protein-conformational diseases. Some examples are familial hypercholesterolemia, osteogenesis imperfecta, retinitis pigmentosa, Fabry disease as well as other diseases where the loss-of-function phenotype is often accompanied by an accumulation of cellular deposits and therefore a gain-of-toxic-function phenotype. In the case of a myopathy known as Paget's disease, intracellular accumulation of aggregates is caused by missense mutations of the p97 (Wheil et al., 2006), gene which affects the ability of cells to degrade misfolded proteins, especially those delivered into the cytosol from the ER lumen. CTFR mutants in cystic fibrosis, on the other hand, illustrate an overzealous "quality control", where biologically active mutants cannot leave the ER. Diseases can also be caused by defects in the intracellular transport mechanisms, such as in bleeding disorders caused by mutations in the carbohydrate-binding sorting receptor ERGIC53. In this case, the mutant is

unable to properly package the blood coagulation factors V and VIII into COPII vesicles for anterograde trafficking out of the ER (Nichols et al., 1998).

### ***1.3 The ER-Golgi Intermediate Compartment***

After their correct folding, newly-synthesized soluble and membrane-bound secretory proteins leave the ER at the specialized domains of the ER membrane, known as ER exit sites (ERES). The ER export is mediated by vesicles (Sheckman and Orci, 1996) directed to the Golgi via the ER-Golgi Intermediate Compartment (Hauri et al., 2000a). Vesicle budding is mediated by a cytosolic complex consisting of the small GTPase Sar1p and the heterodimeric protein complexes Sec23p-Sec24p that form the coatomer COPII. Another type of coat proteins known as COPI have been shown to be involved in the trafficking of proteins between the ER and Golgi complex. These COPI coated vesicles mediate retrograde Golgi to the ER transport and their involvement in anterograde transport is still being debated (Shima et al., 1999).

In mammalian cells (but not in yeast) the transport between the ER and the Golgi complex involves a collection of membranes and highly mobile tubulovesicular clusters called the “ER to Golgi Intermediate Compartment” (ERGIC). This was initially defined following the identification of a 53 kDa membrane protein (ERGIC53) that was predominantly localized to these membranes (Schweizer et al., 1988; Hauri et al., 2000b) and demonstrated as promoting the ER export of a subset of glycoproteins, i.e. the coagulation factor V and VIII (Nichols et al., 1998; Moussali et al., 1999; Hauri et al., 2000b). The dynamic nature and functional role of the ERGIC have been debated for long time. Initially, it was proposed that the ERGIC was a specialized domain of the ER (Sitia

and Meldolesi, 1992) or the cis-Golgi (Mellman and Simons, 1992), however at this time two major hypothesis regarding the pleiomorphic origin of the ERGIC exist. According to the maturation model, the ERGIC clusters are mobile transport complexes that are formed by fusion of the COPII vesicles and carry secretory cargo from the ER to the Golgi complex. Adversely, according to the stable compartment model, ERGIC represents a stationary organelle in which ER-derived cargo is first shuttled from the ER-exit sites to pre-existing ERGIC clusters in a COPII-dependent step and from there to the Golgi in a second vesicular transport step. The sorting in the ERGIC involves COPI vesicles. COPI vesicles play a well-established role in retrograde traffic from both the ERGIC and the Golgi (Klumperman et al., 1998; Letourner et al., 1994) back to the ER. Moreover, COPI vesicles seem to have a role in post-ERGIC anterograde traffic (Pepperkok et al., 1993). COPI might only be necessary indirectly, because it recycles cargo receptors and other factors required for anterograde transport, however an anterograde budding function of COPI at the ERGIC has to be considered (Shima et al., 1999). Therefore ERGIC seems to play a very important role as a sorting station for anterograde and retrograde protein traffic between the ER and the Golgi complex, however in light of this observation we must now question if ERGIC's function goes beyond that of sorting. Another function of ERGIC is the initial concentration of secretory cargo, such as pancreatic enzymes, that leave the ER (Martinez-Menarguez et al., 1999). Increasingly mounting evidence indicates that the ERGIC is also involved in conformational-based quality control of proteins and protein folding (Breuza et al., 2004). Specific post-translational modifications associated with this subcompartment are not known.



### ***1.4 The Golgi apparatus***

The Golgi apparatus is localized around the centrosome (Rambourg and Clermont, 1990) and actively maintained there by its interaction with microtubule array (Lippincott-Schwartz, 1998). It is comprised of stacks of cisternae interlinked by vesicles and tubular networks and is organized into three distinct polarized domains: the cis-Golgi, the medial-Golgi and the trans-Golgi network (TGN). It is primarily involved in O-linked glycosylation events and N-linked oligosaccharide modifications. A highly organized set of glycosyl transferases, glycosidases, and nucleotide- or lipid-linked glycosyl donors and transporters cooperate to produce these modifications. Each component of this protein modification machinery has a characteristic distribution within the Golgi complex and therefore several Golgi enzymes such as Mannosidase I for the cis-golgi or  $\alpha$ -2,6-sialyl transferase for the trans-Golgi are commonly used as markers in order to identify different sub-compartments. The cis-Golgi network receives proteins from the intermediate compartment and is involved in the retrieval of a subset of proteins back to the endoplasmic reticulum. In the medial-Golgi most of the addition and trimming of carbohydrates takes place. The TGN is the exit side of the Golgi complex and has an essential role in the final glycosylation reactions and the sorting of plasma membrane, lysosomes and secretory proteins to their respective final destinations.

### ***1.5 The homeostasis in the secretory pathway***

The flow of secretory proteins along the secretory pathway is continuous and, particularly with specialized secreting cells, can be massive.

In the meantime it is necessary that the organelle identity is maintained in order to ensure an appropriate compartmentalization of the reactions and an ordered mechanism of protein synthesis, assembly, modification, and delivery. Sophisticated mechanisms of sorting based upon aminoacidic signals make the maintenance of organelle homeostasis possible. The ER has a very hard job in this because all the proteins of the secretory pathway spend a moment of their lives inside this organelle. Proteins residing in the ER, such as chaperones like Bip or calreticulin, can use two distinct localization mechanisms that, in most cases, cooperate with each other; as they exit from the ER in anterograde vesicles, they then are retrieved from a post-ER compartment via a retrograde transport (dynamic retrieval) otherwise they are retained in the ER by exclusion from transport vesicles (static retention). The best characterized mechanism of retrieval functions thanks to at least three specific signals.

Most soluble ER-resident proteins contain a carboxy-terminal tetrapeptide sequence, KDEL, Lys-Asp-Glu-Leu. The addition of this sequence to the carboxy-terminus of soluble secreted proteins results in their accumulation in the ER while their removal from the C-terminus of ER luminal proteins results in their secretion. This data implies that such a motif is necessary and sufficient for ER localization (Munro and Pelham, 1987; Nilsson et al., 1989; Pelham, 1988; Jackson et al., 1990). The KDEL sequence is thought to retrieve proteins from the ERGIC or the Golgi complex to the ER (Dean and Pelham, 1990). This retrieval is mediated by the cis-Golgi localized membrane receptor Erd2p which binds to the KDEL sequence in the late IC and the cis-Golgi, and releases the KDEL proteins in the ER (Semenza et al., 1990). Two signals are described as used to retrieve integral membrane proteins to the ER. Type I ER-resident membrane proteins have cytosolic KKXX or KXKXX (dilysine) motifs at their carboxy termini. Direct binding of COPI to the dilysine motif of ER

integral membrane proteins permits them to be retrieved to the ER (Cosson and Letourneur, 1994). Some type II ER-resident membrane proteins have a cytosolic ER localization signal consisting of two arginine residues (RR) often located within the first five amino-terminal residues of the protein. Arg-based signals occur in polytopic membrane proteins that are subunits of membrane protein complexes such as ATP-sensitive ( $K_{ATP}$ ) channels and sulphonylurea receptor proteins, and maintain improperly assembled subunits in the ER by retention or retrieval until masked as a result of heteromultimeric assembly. Recognition by the coatomer COPI is the mechanism that explains the ER retrieval (Michelsen et al. 2005).

### ***1.6 ER export signals***

When the neosynthesized proteins are properly folded they have to leave the ER to reach their final destination. The mechanism of protein export from the ER remains controversial. According to the bulk-flow model, secretory proteins contain no ER-export signals and as such are packaged into transport vesicles by default. However recent evidence suggests that newly synthesized proteins destined for transport out of the ER are specifically concentrated at the ERES. This alternative mechanism implies the presence of sorting signals and a machinery for signal decoding (Kuehn et al., 1998). Recent studies have identified specific residues or sequence motifs on transmembrane cargo that directly bind the components of COPII complex. For soluble secretory cargo proteins that cannot be bound directly by coat subunits no consensus has been described, however evidence suggests that transmembrane receptors might link certain luminal cargo to COPII. Transport of the vesicular stomatitis virus glycoprotein (VSV-G) has served as a model for the study of secretory protein export from the ER. VSV-G is a type I transmembrane protein that traffics to the cell

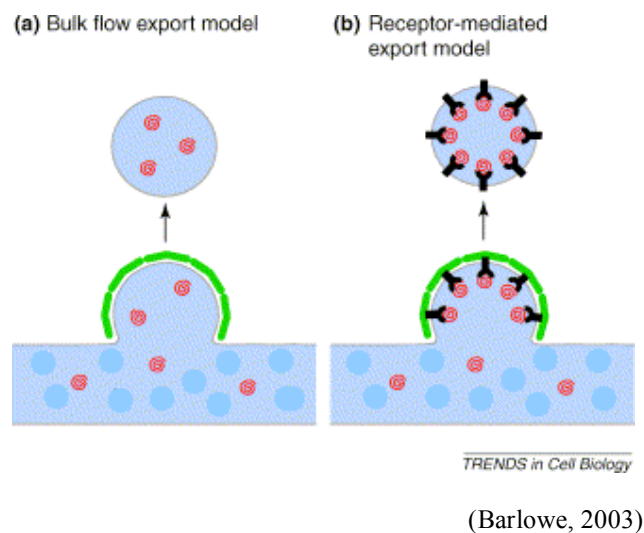
surface and, abundantly expressed in VSV-infected cells, is concentrated into ER-derived transport vesicles (Doms et al., 1988). Within its C-terminal tail, a conserved YXDXE motif is necessary for the efficient export of VSV-G from the ER (Nishimura and Balch, 1997; Sevier et al., 2000). This so-called di-acidic sequence (DXE) motif is found in many other secretory proteins that are efficiently exported from the ER including the Kir2.1 potassium channel protein (Ma et al., 2001) and the yeast membrane proteins Sys1p and Gap1. Moreover, Sys1p depends upon its di-acidic residues for direct binding to Sec23-Sec24 (Votsmeier and Gallwitz, 2001) and Gap1p requires its di-acidic motif to form pre-budding complexes with Sar1 and Sec23-Sec24 (Malkus et al., 2002).

However, there exist many other membrane proteins that are efficiently exported from the ER and do not contain apparent di-acidic motifs. Other types of transport signals have been identified in membrane cargo that exit the ER. These generally consist of a pair of hydrophobic residues and are described as di-aromatic or di-hydrophobic motifs. For example, the type I transmembrane protein ERGIC53 possesses, within its cytoplasmic tail, a conserved pair of aromatic residues (FF) at the extreme C-terminus necessary for transport from the ER and evidence suggests that these terminal residues play a role in binding to the COPII subunits (Kappeler et al., 1997). There is some flexibility in this C-terminal signal as other bulky hydrophobic aminoacids can act as substitutes. Other di-aromatic motifs (FF, YY or FY) are found in a similar position in membrane proteins that exit the ER such as the p24 family (Fiedler et al., 1996) and the Erv41-Erv-46 complex (Otte and Barlowe, 2002). A third class of ER export motif has been described as the sequence RK(X)RK in the N-terminal cytosolic tail of Golgi resident glycosyltransferases which is required for these type II membrane proteins to exit the ER. This di-basic motif is located proximal to the transmembrane border and directly interacts with the

COPII component Sar1 (Giraudo and Maccioni, 2003). Yet a mechanism involving 14-3-3 proteins has been described as affecting the cell surface expression of several unrelated cargo membrane proteins such as the class-II-associated invariant chain lip35 (Kuwana et al., 1998), the KCNK3 subunits (O’Kelly et al., 2002), the assembled  $K_{ATP}$  channel  $\alpha$  subunits (Yuan et al., 2003) and HLA-F (Boyle et al., 2006). 14-3-3 proteins promote the ER exit by binding to phosphorylated residues in the cytosolic domain of transmembrane proteins and in this way, overriding the ER localization dictated by an arginine-based signal of ER retention. Finally, it has been demonstrated that a C-terminal valine is required for the transport to the cell surface of type I transmembrane proteins such as proTGF- $\alpha$ , MT1-MMP (Ureña et al., 1999), CD8 $\alpha$  (Iodice et al., 2001), which suggests that C-terminal valines are general determinants of the subcellular location of cell surface transmembrane proteins (this will be considered more in depth in paragraph 2.1.1).

Other than transmembrane cargo proteins, which are potentially directly accessible to COPII subunits, there exist a variety of soluble secretory proteins that are efficiently exported from the ER and are not able to directly contact the COPII coat. Two non-exclusive models, known as the “bulk flow” and “receptor-mediated” export models, have been described to explain the export of soluble cargo from the ER (fig. 2). A “bulk flow” process appears to operate in the export of amylase and chymotrypsinogen from the ER of pancreatic exocrine cells (Martinez-Menarguez et al., 1999). The receptor-mediated model hypothesizes that the export of soluble cargo from the ER is an active process that concentrates cargo into ER-derived vesicles. In this model, transmembrane cargo receptors would link luminal cargo to the COPII coat. For example, it has been demonstrated that ERGIC53 functions as a transport receptor for several soluble glycoproteins including blood coagulation factors, cathepsin-Z and alpha1-

antitrypsin (Nichols et al., 1998; Appenzeller et al., 1999; Nyfeler et al., 2008). It has been shown that ERGIC53 interacts with Factor VIII in a calcium-dependent way. Besides, transmembrane proteins cycling between the ER and the Golgi compartments would recognize and bind to specific sites within distinct soluble cargo molecules; possible changes in luminal pH and  $\text{Ca}^{2+}$  concentration within distinct membrane compartments could regulate receptor–cargo interactions.



**Figure 2. Bulk flow and receptor-mediated models for export of soluble secretory cargo from the ER. a)** In the bulk-flow model, soluble cargo molecules depart in vesicles at a concentration equal to that found in the ER lumen. **b)** The receptor-mediated export model results in a concentrative sorting of soluble cargo in the vesicles.

## CHAPTER II

### **2. The role of the GRASPs in the C-terminal valine-dependent transport of the human glycoprotein CD8 $\alpha$**

#### ***2.1 Introduction***

##### ***2.1.1 The C-terminal valine motif***

There is some evidence that a carboxy-terminal valine residue at the cytosolic tail of some cell surface transmembrane proteins is an anterograde signal. This signal is part of a PDZ-binding motif present in several receptors and channels.

Deletion or substitution of the valine residues in proTGF $\alpha$  and MT1-MMP proteins results in a lack of surface expression, ER accumulation and proTGF $\alpha$  ectodomain shedding in the culture medium (Ureña et al., 1999).

In the non-classical MHC class I molecules HLA-F, the C-terminal valine also functions as an ER export motif; both a mutant deleted of the C-terminal valine and a mutant with its valine substituted for a serine are retained in the ER (Boyle et al., 2006). Finally, the deletion of C-terminal valine residues in FXYD7, a brain specific protein that associates with the Na,K ATPase isozyme, significantly delayed and decreased the O-glycosylation processing of FXYD7 and retarded the rate of its cell surface expression.

C-terminal valine's importance is also demonstrated by another kind of experimental approach that used the protein ERGIC53 as a reporter. Mutants of a glycosylated variant of human ERGIC53 in which the dilysine signal was replaced by alanines to prevent recycling were generated. A

single valine in -1 position substituted for the FF motif was sufficient to accelerate the transport of the reporter. It was also demonstrated that the valine signal is position, but not context, dependent (Nufer et al., 2002) and requires a minimal tail length which was demonstrated to be 19 aminoacids for the stem cell factor Kit ligand, another transmembrane protein carrying a C-terminal valine (Pahule et al., 2004).

How the C-terminal valine is decoded and what proteins are involved is still under investigation. In vitro binding assays demonstrate that the C-terminal valine interacts with coat complex COPII, particularly with the Sec24Cp (Nufer et al., 2002) and Sec23 (Boyle et al., 2006) proteins. These interactions and the ability of a C-terminal valine to efficiently substitute the FF ER-export signal of the human ERGIC-53, implicate that the C-terminal valine could act at the exit from the ER but does not exclude other effects on the other steps of the secretory pathway. Moreover, the cytosolic tail of proTGF $\alpha$  binds the PDZ-motif-containing proteins (PDZ proteins) syntenin and p59/GRASP55 in a C-terminal valine dependent fashion (Fernandez-Larrea et al., 1999; Kuo et al., 2000) and the binding to the PSD-95 PDZ protein has been correlated to the cell surface expression of K<sup>+</sup> channel proteins (Tiffany et al., 2000).

### 2.1.2 The golgins

The golgins are a variegated family of proteins that only have the Golgi localization, large regions of coiled-coil, and, for many members, the capacity to interact with small GTPases in common (some examples in fig. 3). The diversity amongst the golgins is reflected in the wide range of functions that they carry out.

One of the most-characterized functions of golgins is their role in membrane tethering events such as those demonstrated for the ternary complex p115, GM130 and giantin (Alvarez et al., 2001). Other golgins



perform roles that contribute to efficient transport to the Golgi complex. For example, Bicaudal-D1 and Bicaudal-D2 have recently been shown to be involved in the tethering of vesicles and possibly of some Golgi membranes to the microtubule cytoskeleton (Matanis et al., 2002).

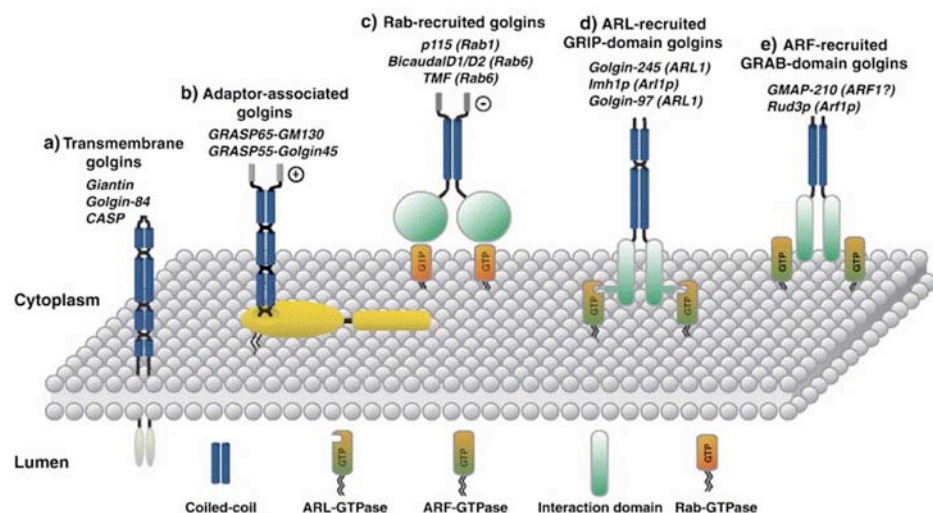
The Golgi Reassembly Stacking Proteins (GRASPs) were identified by *in vitro* assays as factors required for the stacking of Golgi cisternae (Barr et al., 1997; Shorter et al., 1999). There are two isoforms in vertebrates, GRASP65 and GRASP55. GRASP65 is localized in the cis-Golgi, while GRASP55 is predominantly at the medial Golgi (Barr et al., 1997; Shorter et al., 1999). Both proteins are associated with the membrane on the cytosolic face of Golgi cisternae by N-terminal myristoylation.

The GRASPs appear to play important roles in a number of cellular processes. In mammalian cells undergoing mitosis, GRASP65 is phosphorylated by the Cdk-Cyclin B kinase (Lowe and Barr, 2007), whereas GRASP55 is phosphorylated in a pathway involving mitogen-activated protein kinase kinase (MEK1) (Feinsten and Linstedt, 2007). These phosphorylation events modify the biochemical and functional properties of the GRASPs and contribute to mitotic fragmentation. Perturbing GRASP65 activity inhibits Golgi fragmentation at the G2/M transition and this lack of Golgi breakdown prevents cells from progressing through mitosis (Sütterlin et al., 2005). Moreover, it has recently been demonstrated that phosphorylation of GRASP55 at specific tyrosine is necessary for Golgi fragmentation *in vitro* and mitotic entry *in vivo* (Duran et al., 2008).

GRASP65 and GRASP55 play important roles in regulating Golgi structure thanks to their interaction with other members of the Golgins family. GRASP65 binds to GM130 and to the vesicle tethering protein p115 (Lowe and Barr, 2007). GRASP55 is a specific binding partner of the medial-Golgi localized golgin-45. Disruption of this complex by the

depletion of golgin-45 results in dispersal of the Golgi apparatus and inhibition of protein transport (Short et al., 2001).

The GRASPs' involvement in protein trafficking in the secretory pathway is still being debated. There is evidence that they are not directly involved in the trafficking along the secretory pathway of commonly studied reporter proteins such as VSVG and HRP (Duran et al., 2008). However they have been implicated in an “unconventional secretion” of proteins during *Ditctyostilium* and *Drosophila* development (Kinseth et al., 2007). Nonetheless a role of GRASPs in the trafficking of at least selected cargoes can not be ruled out. GRASP65 has been shown to interact with members of the p24 family of cargo receptors that are involved in the recycling between the ER and Golgi (Barr et al., 2001). GRASP55 binds to Transforming Growth Factor- $\alpha$  (TGF- $\alpha$ ) and this interaction is important for the TGF- $\alpha$  expression at the cell surface (Kuo et al., 2000).



(Short et al., 2005)

**Figure 3. Golgins associate with Golgi membranes in a variety of ways.** The golgins are a variegated family of proteins that have only the Golgi localization, large regions of coiled-coil, and for many members, the capacity to interact with small GTPases in common. The diversity amongst the golgins is reflected in the wide range of functions that they carry out.

### 2.1.3 CD8 $\alpha$ , a model system for evaluating the role of the C-terminal valine in anterograde transport

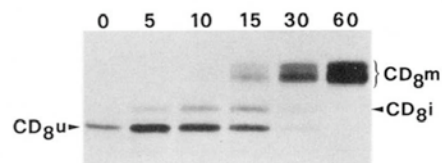
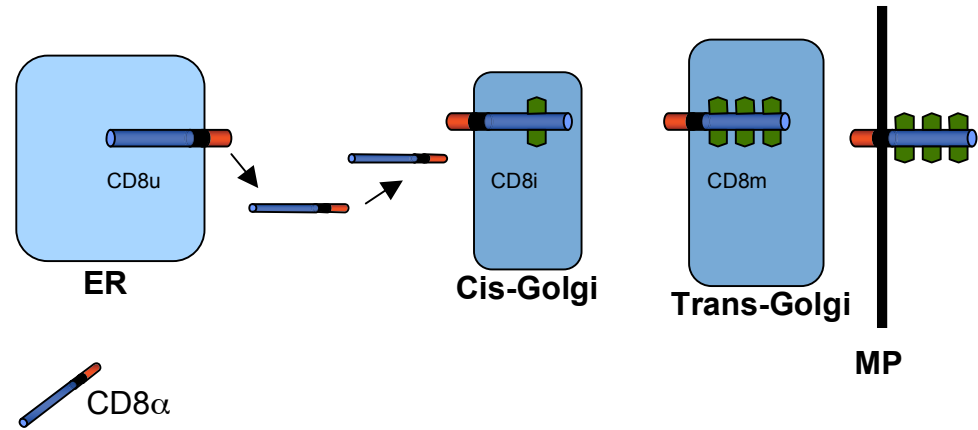
In order to investigate the role of the C-terminal valine in the exocytic pathway, Luisa Iodice et al. (2001) used the lymphocyte CD8 $\alpha$  glycoprotein, an O-glycosylated type I transmembrane protein of the cell surface, as a model system for these reasons: first, the protein moves rapidly from the ER to the plasma membrane and its post-translational modifications are well documented (Pascale et al., 1992); second, progress along the pathway can be monitored by metabolic labeling, because specific forms of the protein occur in the ER, the cis-Golgi and the trans-Golgi complex (Pascale et al., 1992).

The newly synthesized protein migrates as a 27-kDa unglycosylated form known as “CD8u” on SDS-PAGE in reducing conditions. When the protein reaches the cis-Golgi it is initially O-glycosylated and forms an intermediate transient 29-kDa precursor, CD8i. Afterwards the protein is terminally O-glycosylated in the trans-Golgi complex and on SDS-PAGE it appears as a 32-34 kDa mature doublet, CD8m. Finally the CD8m moves to the plasma membrane (fig. 4).

The cytosolic tail of CD8 $\alpha$  was extensively modified at the carboxyl terminus and the mutants were transiently transfected in HuH7 cells. Immunofluorescence analysis of the intracellular distribution of the mutants showed that the mutants lacking the C-terminal valine presented a labeling of the nuclear envelope and a more pronounced Golgi labeling if compared with the wild-type distribution of CD8 $\alpha$ . A pulse-chase experiment showed that the mutations affecting the carboxyl-terminal valine did not block terminal glycosylation and the transport of CD8 $\alpha$  along the exocytic pathway but did decrease the rate of CD8 $\alpha$  glycosylation. The delayed transport rate could be due to the misfolding of

the mutants. The CD8 $\alpha$  folding in the ER results in the formation of homodimers stabilized by disulfide bridges. An experiment of immunoprecipitation and SDS-PAGE in nonreducing conditions showed that the mutant and the wild-type CD8u forms contained the same relative amounts of dimers after a short pulse. Therefore we can conclude that the valine does not play a role in the folding and dimerization of the glycoprotein.

Finally, an experiment of cell fractionation has proven that the C-terminal valine does not play a role in the transport from the trans-Golgi compartment to the plasma membrane and therefore the absence of the carboxyl-terminal valine specifically impairs the transport step between the ER and the IC. This data demonstrates that the C-terminal valine is required for the transport of glycoprotein CD8 $\alpha$  from the ER to the IC. However which molecular mechanisms are actually involved in decoding the C-terminal valine remains unresolved and is currently being investigated in the laboratory of Prof. Bonatti.



**Figure 4. A schematic representation of the intracellular trafficking of the human glycoprotein CD8α.** The newly synthesized protein migrates as a 27-kDa unglycosylated form known as “CD8u” on SDS-PAGE in reducing conditions. When this reaches the cis-Golgi it is initially O-glycosylated and forms an intermediate transient 29-kDa precursor, CD8i. Later the protein is terminally O-glycosylated in the trans-Golgi complex and on SDS-PAGE it appears as a 32-34 kDa mature doublet CD8m. Finally, CD8m moves to the plasma membrane.

#### 2.1.4 Experimental background

It has been suggested that C-terminal valine motifs interact with the COPII subunits (Nufer et al., 2002; Boyle et al., 2006) on the basis of in vitro binding assays. However, some evidence show that PDZ-motif-containing proteins (PDZ proteins) are directly involved in binding C-terminal valine-containing motifs. The cytosolic tail of proTGF $\alpha$  binds the PDZ protein syntenin and p59/GRASP55 (Fernandez-Larrea et al., 1999; Kuo et al., 2000). It has been proven that GOPC, a novel PDZ protein resident in the Golgi complex, plays a role in the transport of the C-terminal bearing receptor FZD8 to the plasma membrane by interacting with its cytosolic tail. Furthermore, the mutation of valine to alanine in the C-terminus of FZD8 significantly reduces the interaction and the deletion of the Ser/Thr-X-Val motif completely abolishes the interaction (Yao et al., 2001).

Recent data obtained by G. D'Angelo et al. in the laboratory of Prof. Bonatti, and not yet published, gives evidence that CD8 $\alpha$  directly binds to the golgins GRASP65 and GRASP55 in a C-terminal valine-fashion and that these interactions are necessary for the transport of the glycoprotein to the plasma membrane.

Lysates obtained from parental FRT cells and from FRT cells stably transfected (fig. 6a,b) or HuH7 cells transiently transfected (fig. 6d) with the CD8 $\alpha$  constructs indicated and schematically represented in fig. 5, were subjected to immunoprecipitation with an anti-CD8 antibody. Efficient coprecipitation of GRASP65 and GRASP55 was observed only in the immunoprecipitates of cells expressing CD8 $\alpha$ wt and the mutant  $\Delta$ 17 bearing a C-terminal valine,  $\Delta$ 17+. Moreover GM130, which is known to strongly bind to GRASP65, also coimmunoprecipitated in a C-terminal-valine-dependent manner.

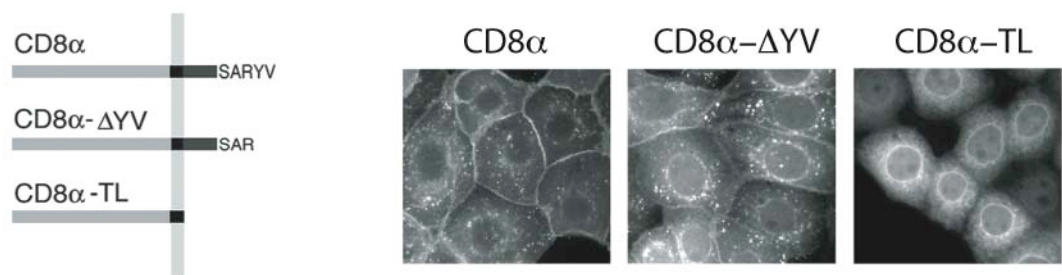
In order to determine if CD8 $\alpha$  directly interacts with GRASP65 and GRASP55, Far Western blotting assay was performed. Purified GST protein and the fusion proteins bearing the cytosolic tails of CD8 $\alpha$ wt and CD8 $\alpha$  $\Delta$ YV, GST-CD8 $\alpha$ wt and GST-CD8 $\alpha$  $\Delta$ YV, were analyzed by SDS-PAGE and transferred to nitrocellulose filters. The filters were overlaid with purified His-tagged proteins GRASP65, GRASP55, a mutant of GRASP65 lacking the two PDZ domains  $\Delta$ PDZ-GRASP65, and a non-related protein as a negative control FAPP2 (fig 6c). The bound proteins were detected with an anti-His antibody. His-GRASP65 and His-GRASP55 bound to GST-CD8 $\alpha$ wt but not to GST-CD8 $\alpha$  $\Delta$ YV. As expected, no specific interactions were observed between the GST proteins and His-FAPP2. Nor was there binding detected with the mutant of GRASP65,  $\Delta$ PDZ-GRASP65. Therefore, the C-terminal valine of CD8 $\alpha$  triggers direct binding to the golgins GRASP65 and GRASP55 and the interaction occurs via PDZ domains.

To determine which transport step the interaction between CD8 $\alpha$  and GRASP65 occurs at, lysates of cells stably expressing CD8 $\alpha$  and pulse-labeled with  $^{35}$ S-Cys/Met were co-immunoprecipitated with an anti-GRASP65 antibody (fig 6e). The product of the immunoprecipitation was re-suspended and immunoprecipitated with an anti-CD8 antibody. The CD8u form of CD8 $\alpha$  was highly enriched, indicating that the interaction with GRASP65 is transient and occurs before CD8 $\alpha$  reaches the Golgi complex. This has been confirmed by another experiment based on the ability of the fungin drug Brefeldin A to synchronize the ER exit of CD8 $\alpha$  and CD8 $\Delta$ YV in stably expressing FRT. BFA has been shown to induce a breakdown of the Golgi complex, the redistribution of most of the Golgi proteins into the ER and the accumulation in the ER of newly synthesized proteins that normally are destined to be secreted or located downstream in the secretory pathway (Klausner et al., 1992; Ward et al., 2001). The Golgi

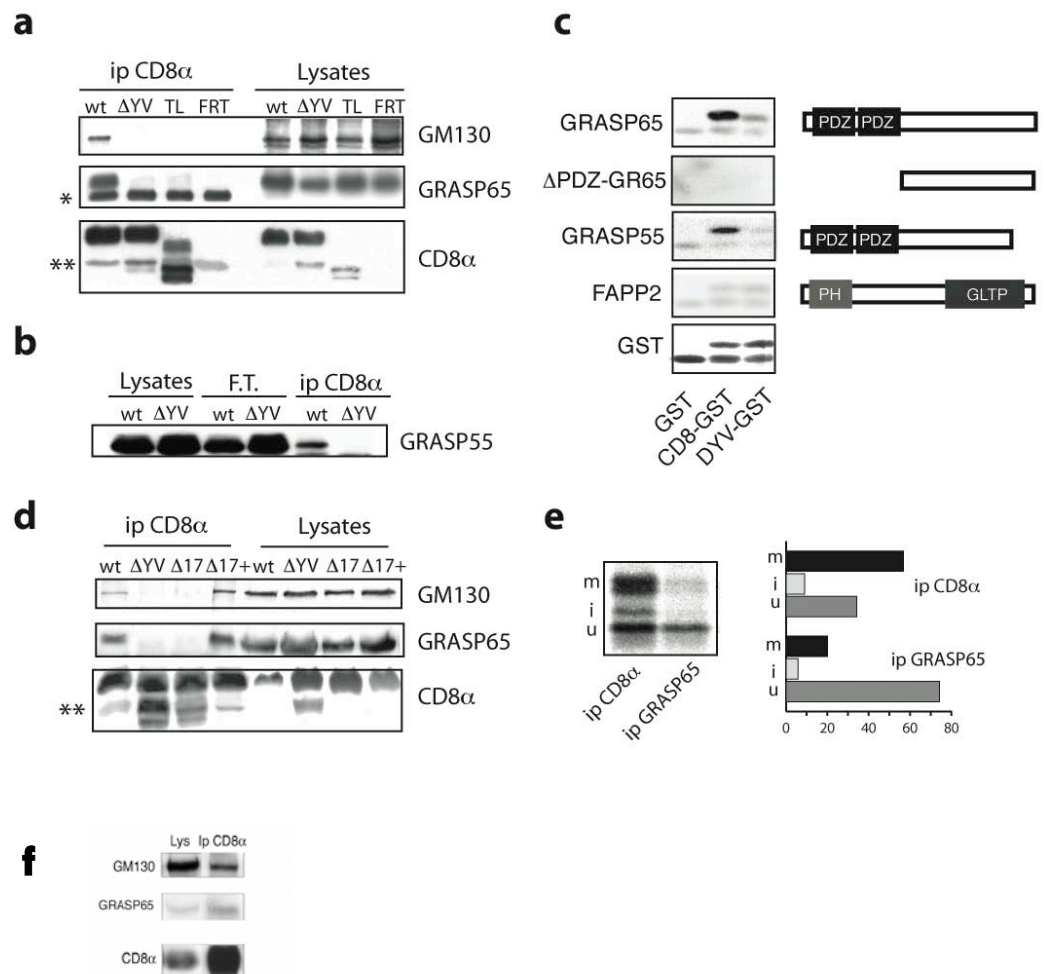
matrix proteins such as GRASP65, GRASP55 and GM130 are, instead, largely localized to peripheral structures known as Golgi/IC remnants, which are close to and communicate with the ERES (Ward et al., 2001). As shown in fig. 7, in the absence of BFA, a fraction of GRASP65, CD8 $\alpha$  and CD8 $\Delta$ YV are co-localized in the Golgi complex and centrally located IC elements. After BFA treatment, the colocalization was completely lost, and the GRASP65, CD8 $\alpha$  and CD8 $\Delta$ YV labeling was dispersed into numerous puncta throughout the cytoplasm. However, upon BFA washout where the ER export is rapidly restored and the Golgi complex reassembles, CD8 $\alpha$  again colocalized with GRASP65 in dispersed puncta after 10 minutes of BFA washout and it completely recovered its steady-state distribution after 30 minutes. In contrast, the CD8 $\Delta$ YV mutant was retained in the ER and started to colocalize with GRASP65 only at a 30 minute BFA washout, confirming an early defect in the anterograde transport.

Finally, it was assessed whether the interaction discovered occurs physiologically. Lysates of HBP-ALL, human lymphocyte cell line expressing both GRASP65 and CD8 $\alpha$ , were subjected to a coimmunoprecipitation experiment by an anti-CD8 antibody. As shown in fig.6f, endogenous CD8 $\alpha$  coimmunoprecipitated with GRASP65 and its interactor GM130.

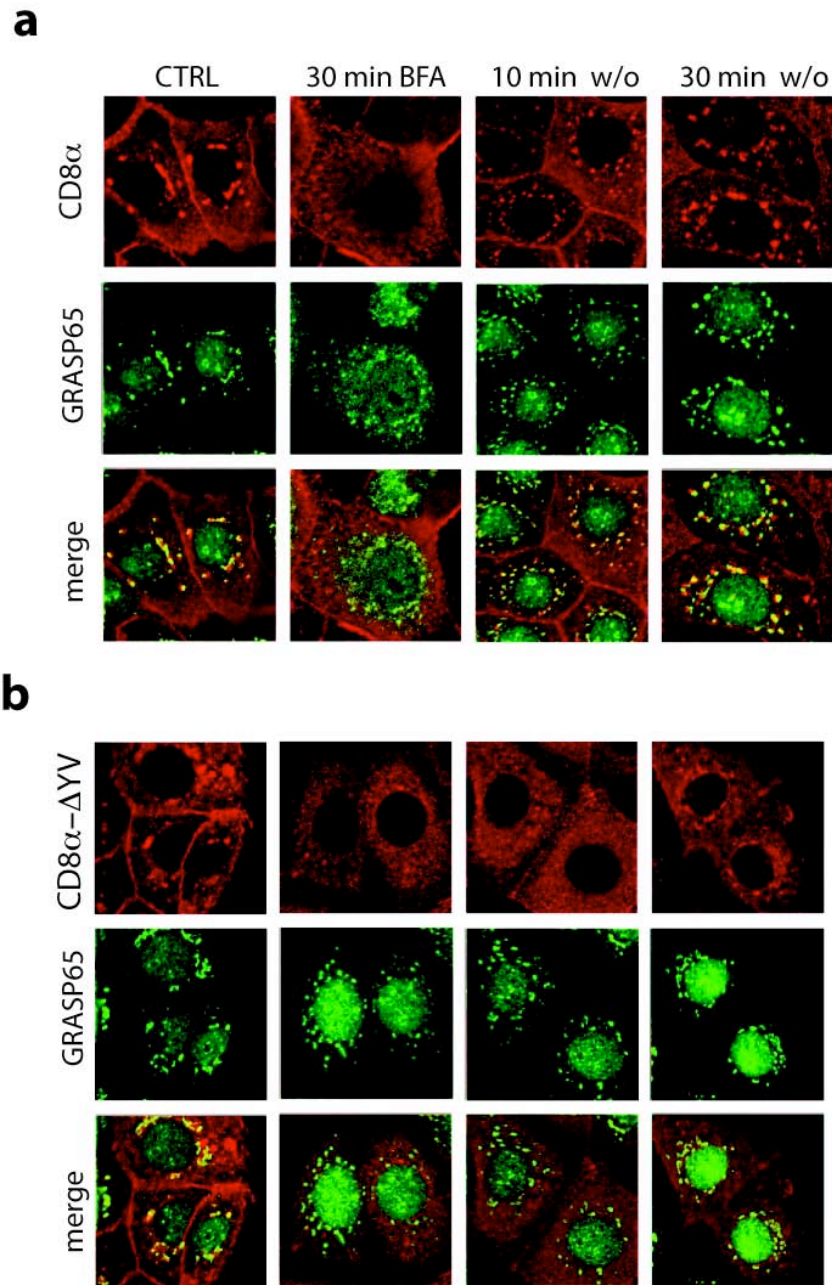




**Figure 5. Schematic representation and intracellular distribution of the constructs used.** The constructs CD8αwt, CD8α lacking the C-terminal tyrosine-valine CD8α-ΔYV and CD8α lacking the entire cytosolic tail, CD8α-TL are schematically represented (left panel). FRT cells stably expressing the CD8αwt, CD8α-ΔYV and CD8α-TL proteins analysed by immunofluorescence (right panel). (G. D'Angelo et al., unpublished data).



**Figure 6. CD8 $\alpha$  binds GRASP65 and GRASP55 in a C-terminal-valine-dependent fashion** **a,b)** Cell lysates obtained from parental FRT cells and from FRT cells stably expressing the different CD8 $\alpha$  constructs were subjected to immunoprecipitation with an anti-CD8 antibody (IP CD8 $\alpha$ ). The immunoprecipitated products were analyzed by SDS-PAGE, followed by immunoblotting performed with the antibodies indicated on the right. **c)** Purified protein GST, GST-CD8wt and GST-CD8 $\Delta$ YV were resolved on 12% electrophoresis gel and transferred onto a nitrocellulose filter. The filters were incubated with purified proteins His-tagged indicated and the bound proteins were detected with an anti-His antibody. **d)** Huh7 cells were transiently transfected to express the different CD8 $\alpha$  recombinants indicated along the top. Aliquots of total cell lysates were analyzed directly on SDS-PAGE followed by immunoblotting (Lysates), or after immunoprecipitation with an anti-CD8 antibody (IP CD8 $\alpha$ ). The filter was cut into three portions and the blots were developed with the antibodies indicated on the right. **e)** FRT cells stably expressing CD8 $\alpha$  were pulse labeled for 30 min with [ $^{35}$ S]-methionine and cysteine and lysed. the lysate was immunoprecipitated with an anti-GRASP65 antibody. The immunoprecipitated products were resuspended and immunoprecipitated with an anti-CD8 antibody (IP GRASP65 + IP CD8 $\alpha$ ). The CD8u, CD8i and CD8m forms of CD8 $\alpha$  are indicated. **f)** Lysates of HBP-ALL cells were subjected to a coimmunoprecipitation experiment by an anti-CD8 antibody. Endogenous GRASP65 and GM130 coimmunoprecipitated were revealed by western blot. (G. D'Angelo et al., unpublished data).



**Figure 7. The C-terminal valine mediates dynamic colocalization with GRASP proteins.** FRT cells stably transfected with plasmids expressing CD8 $\alpha$  **a)** or CD8 $\Delta$ YV **(b)** were incubated with BFA for 30 minutes and then washed out for the times indicated. The cells were treated for indirect immunofluorescence and analyzed by confocal microscopy. (G. D'Angelo et al., unpublished data).

### 2.1.5 Objectives

The results stated above prompted us to further investigate the physiological significance of the C-terminal valine-dependent interaction between the CD8- $\alpha$  glycoprotein and the GRASPs. Therefore the following phase of my thesis project addresses two main objectives:

- 1) To locate more precisely at which stage of the early secretory pathway the valine signal works by utilizing a VSV-G ts045-based approach
- 2) To assay whether the interactions identified have a role in the transport of the glycoprotein to the cell surface.

## **2.2 Results**

### 2.2.1 The C-terminal valine motif of CD8 $\alpha$ promotes an early transport step between the ER and the Golgi complex

First of all, we asked whether the valine functions as a signal of export from the ER with a mechanism similar to motifs such as the diacidic motif.

In order to address this question we utilized as an experimental system the temperature-sensitive mutant of the glycoprotein VSV-G (Presley et al., 1997), VSV-G ts045 and two mutants that we generated in our lab.

VSV-G ts045 bears a point mutation that makes it unable to correctly fold when expressed at a non-permissive temperature (39°C) and which therefore becomes trapped in the ER by Quality Control system. Upon a shift to 32°C, the protein rapidly folds and moves through the secretory pathway to reach the plasma membrane. VSV-G exits the ER thanks to a COPII-binding diacidic motif. We generated two mutants constituted of the ectodomain and transmembrane domain of VSVG ts045 and bearing the

cytosolic tail of CD8 $\alpha$ , VSVG-CD8 $\alpha$ , or the cytosolic tail of CD8 $\alpha$  lacking the C-terminal Tyrosine and Valine, VSVG-CD8 $\Delta$ YV(fig. 8a).

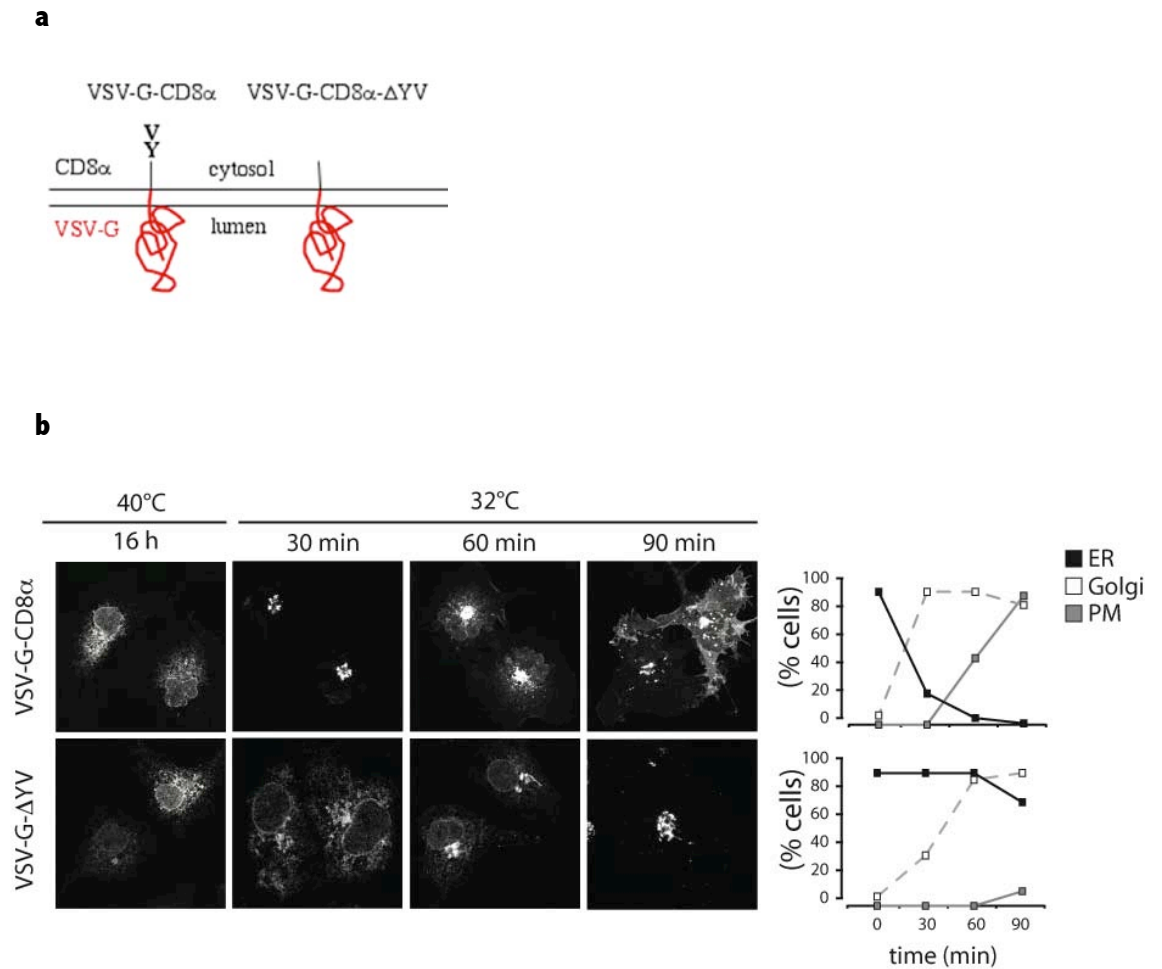
Our first step was to test if the chimera VSVG-CD8 $\alpha$  maintained the characteristics of both the viral protein and CD8 $\alpha$ : the reversible temperature-sensitive phenotype that causes misfolding and retention of VSV-G in the ER and its correct folding and exit from the ER upon a shift to the permissive temperature, and the C-terminal-valine dependent rapid transport to the Golgi complex of CD8 $\alpha$ . Both of the chimerae were expressed by transfection in COS-7 cells incubated at 40°C. The confocal immunofluorescence analysis revealed that at this temperature, both the chimeric forms were retained in the ER. In contrast, upon shifting the temperature to 32°C, VSVG-CD8 $\alpha$  moved quickly to the Golgi complex and from there on to the plasma membrane, whereas VSVG-CD8 $\Delta$ YV showed an accumulation in the ER, a delay in transport to the Golgi complex and a lack of plasma membrane labeling at the 90 minute time point (fig. 8b), in accordance with the previously demonstrated role of the C-terminal valine in the anterograde transport of CD8 $\alpha$ .

And so, in order to test the ability of the C-terminal valine of CD8 $\alpha$  to interact with COPII coat, we performed a 10°C temperature block experiment. At 10°C the recruitment of COPII components to the ERES is allowed, but the following fission step of the nascent carrier is prevented. Under these conditions cargo proteins that can directly interact with COPII components such as VSVG concentrate at the ERES (Lotti et al., 1996). COS7 cells were transiently transfected for the expression of both the chimeric proteins kept at 39°C in order to retain the proteins in the ER and synchronize the export, and then shifted to a 10°C temperature. As shown in fig. 9a, as expected, VSVG accumulated at the ERES at 10°C while VSVG-CD8 $\alpha$  was unable to do so and VSVG-CD8 $\Delta$ YV didn't accumulate at the ERES at 10°C either. Both proteins have a distribution similar to the

VSVG-AXA, a VSVG mutant lacking the diacidic motif, which for this reason was chosen as negative control. This data indicates that the C-terminal valine doesn't work as a COPII-binding motif, which prompted us to investigate the role of this anterograde signal in subsequent steps of transport. Therefore, using the same constructs as described above, we performed a 15°C temperature block; a condition leading to an accumulation of cargo proteins in movement toward the Golgi in the IC. Under this condition VSVG-CD8 $\alpha$  showed a strong accumulation in the IC, similar to the protein VSVG, whereas VSVG-CD8 $\Delta$ YV did not accumulate in the IC and presented a reticular staining as at the 10°C temperature and, similar to the mutant VSVG-AXA which is unable to exit the ER (fig. 9b). These results suggest that the C-terminal valine acts in the step succeeding the ER exit and before arriving at the Golgi complex which is likely to be in the IC.

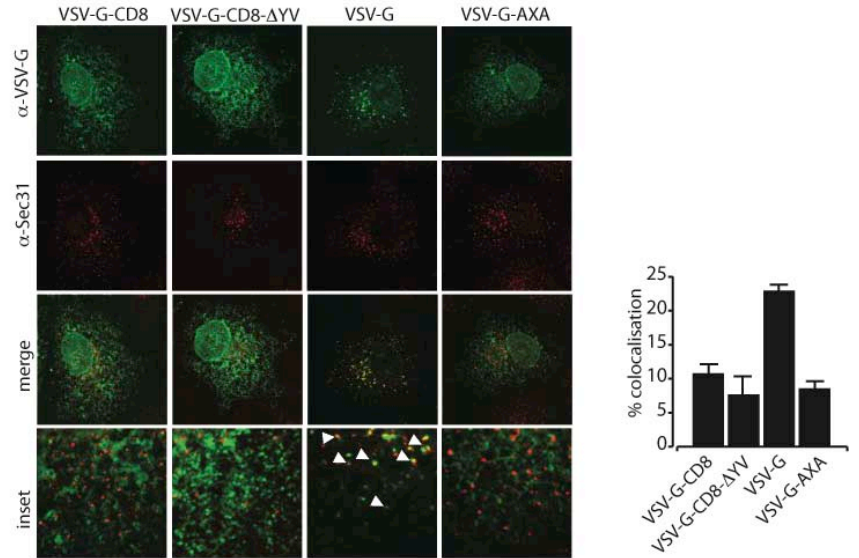
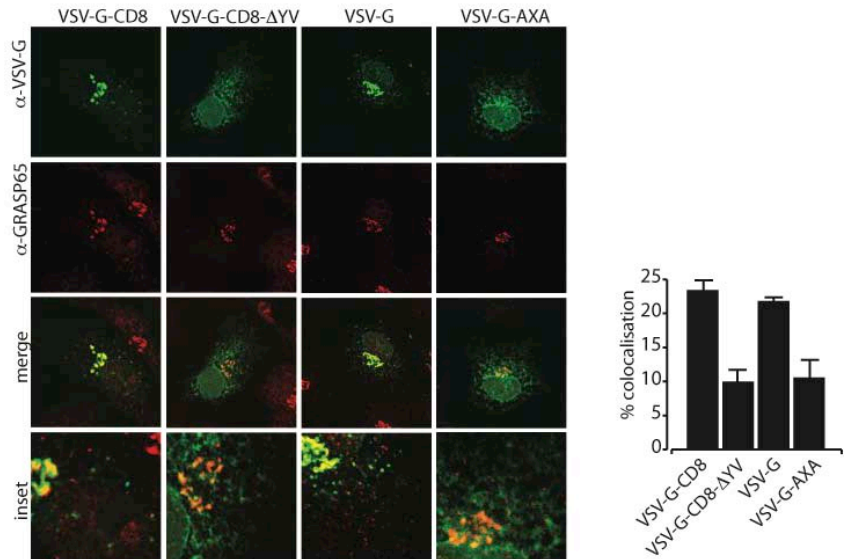
To further validate this hypothesis we performed a budding in vitro experiment. A total microsomal fraction was prepared by differential centrifugation from cells that had been pulse-labeled with <sup>35</sup>S-Cys-Met, and incubated in the presence of an ATP-regenerating system and rat liver cytosol. Microsomes were again recovered by centrifugation, while the vesicles generated during the incubation were collected by ultracentrifugation. Aliquots of the different fractions were then analyzed by SDS-PAGE and western blot (fig.10). We compared the efficiency of the budding of the proteins CD8 $\alpha$ , CD8 $\Delta$ YV and of a CD8 $\alpha$  mutant lacking its entire cytosolic tail, CD8-TL. As a negative control we used a CD8-E19, which is a form of CD8 $\alpha$  with its cytosolic tail substituted with that of the E19 adenovirus protein. This chimera has been shown to have a low rate of ER exit (Stornaiuolo et al., 2003). As positive control we used CD8-K constituted of the ectodomain of CD8 $\alpha$  carrying the KDEL signal at the C-terminus, which has been shown to be efficiently exported from

the ER. As shown in fig. 10, the efficiencies of the budding of CD8 $\alpha$ -wt and CD8- $\Delta$ YV were very similar and truly comparable to the efficiency of the budding of CD8-K. In contrast, as expected, CD8-TL and CD8-E19 had a low budding efficiency. These *in vitro* results confirmed the *in vivo* data: that the C-terminal valine signal does not promote the exit from the ER.



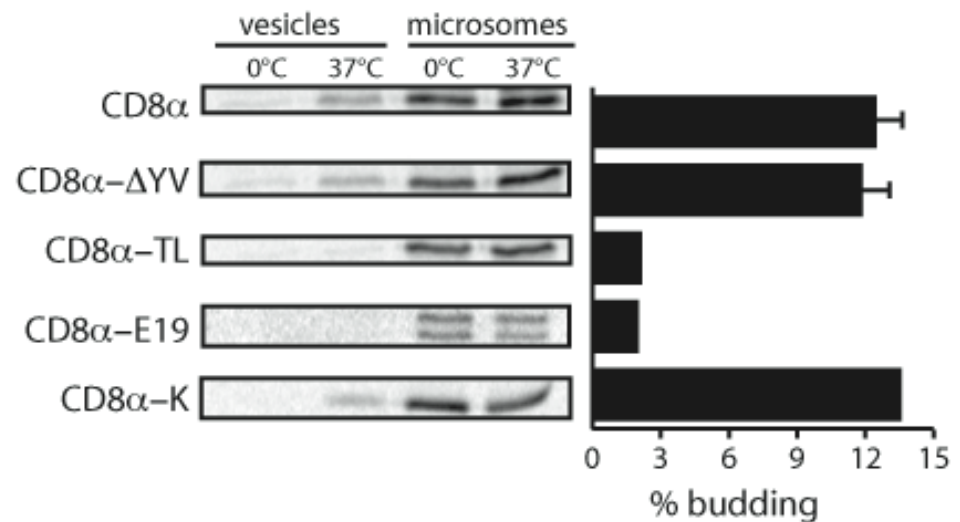
**Figure 8. VSV-G-ts045 based chimeric proteins as a model to thoroughly investigate the role of C-terminal valine in the anterograde transport of CD8 $\alpha$ .** **a)** Schematic representation of the constructs used. **b)** COS-7 cells were transfected with the chimeric constructs VSV-G-CD8 $\alpha$  and VSV-G-CD8 $\Delta$ YV and incubated at 40°C, they were then shifted to a 32°C incubation for the times indicated and analyzed by confocal immunofluorescence microscopy. The accumulation of the proteins in the ER, Golgi complex and plasma membrane are reported in the graphs.



**a****b**

**Figure 9. The C-terminal valine motif of CD8α promotes an early transport step between the ER and Golgi complex.** The VSV-Gts045 based constructs VSV-G-CD8α, VSV-G-CD8ΔYV, VSV-G and VSV-G-AXA were transiently transfected in Cos-7 cells. After 48 hours the cells were incubated for 3 hours at 10°C in **a**, and at 15°C in **b**, they were then treated for indirect immunofluorescence and stained with an anti-VSVG antibody (green) and an anti-Sec31 antibody (red) in **a**, an anti-GRASP65(red) in **b**. The percentages of colocalization were quantified and reported in the graphs.



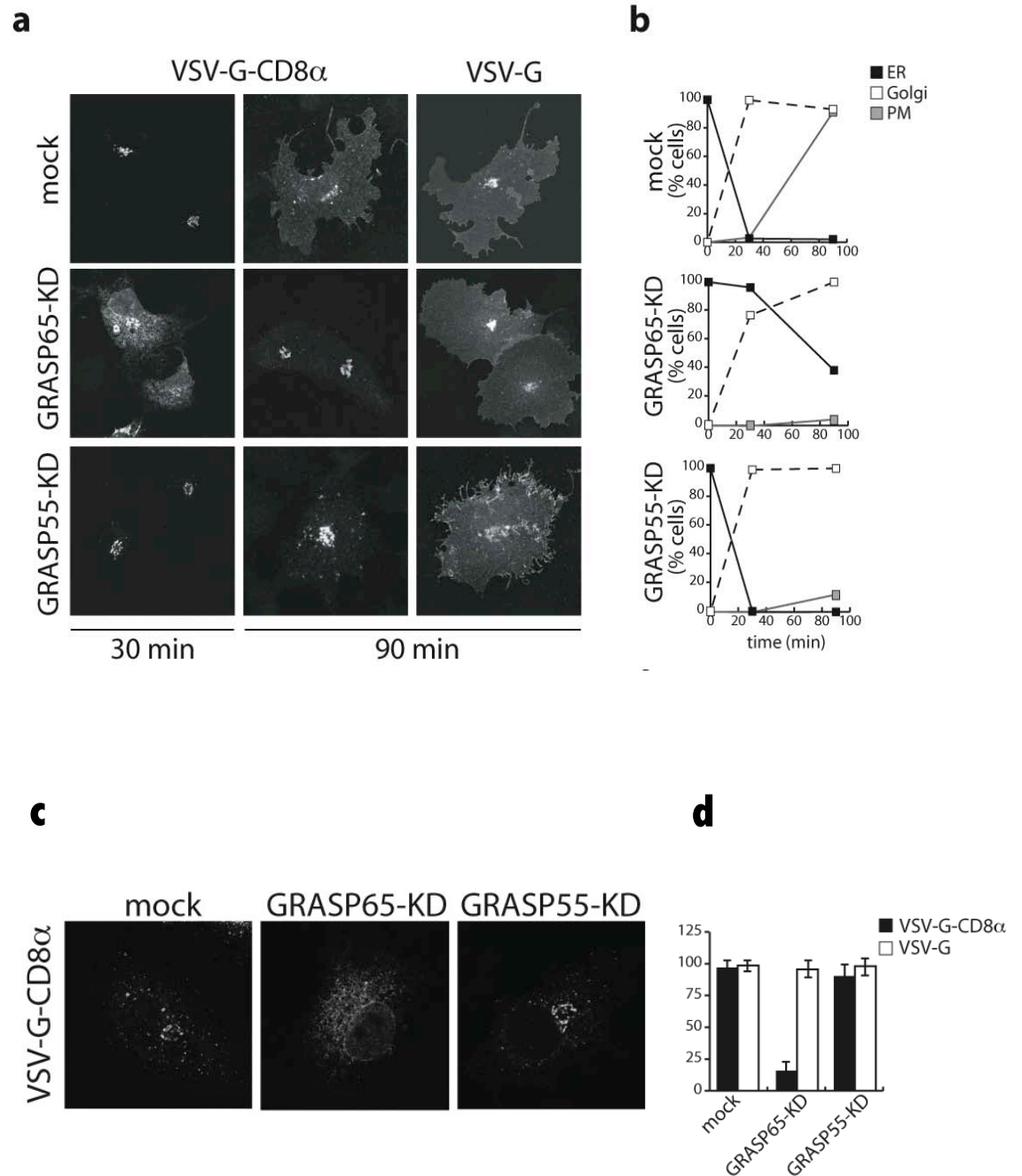


**Figure 10. CD8α and CD8ΔYV show the same rate of ER export *in vitro*.** FRT cells stably transfected for the expression of the proteins CD8α, CD8ΔYV, CD8α -TL, CD8α-E19, CD8α-K were pulse-labeled for 15 minutes and then used as the starting material for the *in vitro* binding assay. On the left, the immunoprecipitated form recovered in the vesicular and microsomal fraction at the end of the 20 minute incubation at 0°C or 37°C. On the right, the quantification of the budding efficiency of each protein recovered in the vesicular fraction at 37°C with respect to the total (vesicular + microsomal) fractions.

### 2.2.2 GRASP65 and GRASP55 are needed for the full rate of transport of CD8 $\alpha$

To determine the role of the C-terminal valine dependent interaction of CD8 $\alpha$  with GRASP65 and GRASP55, we used the RNA interference (RNAi) technique. First we knocked down the GRASP65 and GRASP55 expression in COS7 cells. The cells were then transfected for the VSVG-CD8wt and VSVG-GFP expression, incubated at 40°C and shifted to 32°C for the time indicated in fig. 11(a, b). They were then analyzed by immunofluorescence. At the 30 minute shift, while in the mock transfected cells and in GRASP55-knocked down cells VSVG-CD8 $\alpha$  appeared to have accumulated in the Golgi complex, in the GRASP65-knocked down cells, the protein was mostly distributed to the ER and delayed in its reaching the Golgi complex. After 90 minutes of the 32°C temperature shift, VSVG-CD8 $\alpha$  had reached the plasma membrane in the mock transfected cells while, in contrast, in the GRASP65-knocked down cells the chimera presented a Golgi staining and in the GRASP55-knocked down cells, the chimera was mostly distributed to the Golgi complex and had not yet reached the plasma membrane. These results show that, in absence of GRASP65, the transport of VSVG-CD8 $\alpha$  between the ER and Golgi complex is inefficient, while in the absence of GRASP55, the defect in transport occurs at a later step, which suggests that GRASP55 acts after GRASP65 in the transport. It should be noted that, under the same conditions, the transport of VSVG to the cell surface remained unaffected suggesting that GRASP65 and GRASP55 have specific roles only in the transport of proteins bearing a C-terminal valine. To further investigate these roles, a RNA interference experiment was performed at a temperature shift of 15°C (fig.11c, d). In the GRASP65-knocked down cells, VSVG-CD8 $\alpha$  resulted to be mostly distributed in the ER, showing that the

transport to the IC was inhibited. This distribution did not occur in the GRASP55-knocked down cells and, as expected, nor in the mock transfected cells. These observations support the hypothesis that GRASP65 is needed to enter and proceed through the IC while GRASP55 is needed to enter or proceed through the Golgi complex. Moreover this data is in accordance with the localization of GRASP65 in the IC and the very first Golgi cisterna, while GRASP55 localized mostly in the medial Golgi cisternae.



**Figure 11. GRASP65 and GRASP55 are needed for the full transport rate of CD8 $\alpha$ .** **a)** The expression of GRASP65 and GRASP55 in Cos-7 cells was knocked down by RNAi, the cells were then transfected for the expression of VSV-CD8 $\alpha$  or VSV-G-GFP, incubated at 40°C and then shifted to a 32°C temperature for the times indicated. The percentage of cells showing protein accumulation in the ER, the Golgi, and the plasma membrane for each experimental condition is shown in **b**. **c)** Cos-7 cells, subjected to RNAi as in **a** and then transfected for the expression of VSV-CD8 $\alpha$  were incubated at 40°C and then shifted to 15°C for 3 hours. The percentage of cells showing protein accumulation in the ERGIC for each experimental condition is shown in **d**.

### ***2.3 Conclusions***

This phase of the project was based on a strategy utilizing the temperature-sensitive mutant of the glycoprotein VSV-G, budding in vitro experiments and the RNA interference technique whereby we were able to demonstrate that:

- 1) the C-terminal valine motif of CD8 $\alpha$  promotes an early transport step between the ER and Golgi complex which is not the exit-step from the ER;
- 2) GRASP65 and GRASP55 sequentially decode the C-terminal valine motif of the glycoprotein CD8 $\alpha$  en route to the Golgi complex.

## CHAPTER III

### **3. The role of the C-terminal valine motif in the transport to the plasma membrane of the receptor Frizzled4 and Familial Exudative Vitreoretinopathy (FEVR)**

#### ***3.1 Introduction***

##### **3.1.1 The Frizzled family**

Frizzleds (FZDs) are cell surface receptors with an important regulatory role during embryonic development and in tissue homeostasis in many different organs in the adult. These receptors are termed “frizzled” on the basis of an early phenotype which was observed in the wing of *Drosophila* when the gene of a prominent member of the receptor was inactivated (Chan et al., 1992).

Ten genes encoding FZDs have been identified in the mouse and human genomes, whereas four are known to exist in the *Drosophila* and three have been reported in *C.elegans* (Nusse, 2005). Due to their structure, FZDs are listed as a novel separate family of G-protein coupled receptors (GPCRs), the “Class Frizzled” (Foord et al., 2005). As has been observed for other G-protein coupled receptors, Frizzleds can be considered membrane receptors with three basic regions: the extracellular N-terminus which contains a cysteine-rich domain involved in the binding of the receptor’s ligands, the lipoglycoproteins of the Wnt family (Nusse, 2003), a transmembrane central core composed of seven alpha-helices that span the lipid bilayers, and at least three intracellular loops and a C-terminal “tail” that

communicate with downstream signaling elements found in the cellular cytoplasm (Morris and Malbon, 1999).

FZDs bind several secreted molecules. The primary endogenous agonists are the Wnt proteins, of which there are 19 mammalian forms triggering most of the FZD-mediated signaling pathways.

R-spondin (Nam et al., 2007) and Norrin (Smallwood et al., 2007) can directly bind FZDs and activate downstream signaling. Soluble Frizzled-related proteins (sFRPs) were initially seen as Wnt scavengers that prevented Wnt from binding to FZDs however recent studies support the concept of direct binding to FZD-CRDs followed by receptor activation (Rodriguez et al., 2005). The connective-tissue growth factor (CTGF) can bind the CRD of FZD and inhibit downstream signaling (Mercurio et al., 2004). Other proteins such as Wnt inhibitory factor-1 (WIF-1), Cerberus and members of the Dkkopf family ligands are described as FZD antagonists (Hsieh et al., 2004). There is an apparent, though not adequately characterized, specificity between individual FZDs and their ligands (Kikuchi et al., 2007) further complicated by the existence of several other transmembrane proteins, LRP5/6, RYK and ROR2, that bind FZD ligands and serve as WNT co-receptors (Kikuchi et al., 2007).

Three main signaling pathways are activated by agonist-activated FZDs: the FZD/ $\beta$ -catenin pathway, the FZD/PCP (planar cell polarity) pathway and the FZD/ $\text{Ca}^{2+}$  pathway. In the FZD/ $\beta$ -catenin pathway, agonist stimulation results in the activation of the phosphoprotein Dishevelled (DVL), leading to inhibition of a constitutively active glycogen-synthase kinase 3 which regulates the phosphorylation and destruction of  $\beta$ -catenin. The spared  $\beta$ -catenin is translocated to the nucleus, where it cooperates with TCF/LEF transcription factors to modify gene transcription (Gordon and Nusse, 2006).

In the FZD/PCP pathway, information is transduced via DVL to the small GTPases RHO and RAC and their effectors, ROCK (RHO kinase) and the c-Jun-N-terminal kinase-c-jun-AP1 pathway (Seifert and Mlodzik, 2007).

In the FZD/Ca<sup>2+</sup> pathway, the agonists induce elevation of intracellular calcium levels in a G-protein-dependent manner either directly, through activation of phospholipases (Slusarski, 1997), or indirectly via a decrease in intracellular cyclic GMP (Ma and Wang, 2006), resulting in the activation of calcium-dependent kinases, such as calcium-dependent protein kinase (PKC) and Ca<sup>2+</sup>/calmodulin-dependent protein kinase (Kohn and Moon, 2005).

### 3.1.2 Frizzled4 and familial exudative vitreoretinopathy (FEVR)

Familial exudative vitreoretinopathy (FEVR) is a hereditary ocular disorder characterized by an abrupt cessation of the growth of peripheral capillaries leading to an avascular peripheral retina. This condition may lead to compensatory retinal vascularization which is thought to be induced by hypoxia from the initial avascular insult. New vessels easily break causing exudates and bleeding followed by scarring, retinal detachment and blindness. Although the penetrance of the disease approaches 100%, the phenotype can vary greatly. In the mildest form, affected individuals are asymptomatic and the disease can be diagnosed only by intravenous fluorescein angiography.

The disorder is inherited in autosomal dominant, autosomal recessive and X-linked recessive patterns (de Crecchio et al., 1998), and has been shown to be associated with mutations affecting several genes. X-linked forms of the disease have been described as being associated with mutations in the gene that encode the norrin protein, an extracellular ligand of Frizzled receptors (Chen et al., 1993; Shastri et al., 1995). Mutations in



the gene encoding LRP5, a Wnt co-receptor, have been implicated both in autosomal dominant and autosomal recessive FEVR (Toomes et al., 2004; Jiao et al., 2004). Autosomal dominant forms of the disease have been shown to be caused by mutations in the FZD4 gene (Kondo et al., 2003; Robitaille et al., 2002; Nallathambi et al., 2006). Most of these studies were genetic analysis of FEVR patients however two FZD4 mutants causing the disease have been characterized more specifically (Robitaille et al. 2002). These two mutants were unable to activate  $\text{Ca}^{2+}$  /calmodulin-dependent protein kinase and calcium-dependent protein kinase (PKC). The defect in one case was caused by a deletion in the coding sequence that resulted in deletion of two highly conserved aminoacids which altered the seventh transmembrane domain. In the other mutant a deletion of two nucleotides led to a frameshift and synthesis of a mutant protein with a completely different truncated cytosolic tail. In the second mutant case defective signaling was caused by altered trafficking: the protein is unable to reach the plasma membrane and is retained in the ER (Robitaille et al., 2002). The genetic dominance of this last FEVR allele has also been subsequently explained. Studies based on coimmunoprecipitations of tagged monomers of members of the FZD family have demonstrated that the FZD family, like G-protein-coupled receptors, form specific homo- and hetero-oligomers and that the truncated mutant of FZD4 causing FEVR oligomerizes with wild-type FZD4, retaining it in the ER and inhibiting its signalling (Kaykas et al., 2003).

Interestingly, a genetic variant of the FZD4 gene has been also associated with advanced retinopathy of prematurity (ROP), a disease very similar clinically to FEVR which occurs in infants of short gestational age and low birthweight (MacDonald et al., 2005).

### 3.1.3 Objectives

It should be noted that Frizzled4 and several other members of the FZD family, as well as some plasma membrane proteins discussed above, have a cytosolic tail bearing a C-terminal valine within a PDZ-interacting motif. Interestingly, the mutant FEVR-causing with a different and truncated cytosolic tail (and therefore lacking the C-terminal valines), is retained in the ER. These observations and our previous results with CD8 $\alpha$  prompted us to question whether the absence of the C-terminal PDZ-interacting motif and, in particular, of the C-terminal valine, is the cause of the FZD4 mutant's inability to reach the plasma membrane. In this vein of thinking we decided to:

- 1) investigate the role of C-terminal valine in the transport of FZD4 to cell surface,
- 2) characterize the partners and the molecular mechanisms involved.

## **3.2 Results**

### 3.2.1 The C-terminal valine is required for the efficient transport of FZD4 from the endoplasmic reticulum to the plasma membrane

In order to investigate if the C-terminal valine has a role in the transport of FZD4 to the plasma membrane and identify the transport defect of the mutant, we first mutagenized the cytosolic tail of human FZD4.

The construct HA-FZD4wt encoding for the protein FZD4 with a HA-tag at the N-terminus, was used as a template to generate by site direct mutagenesis the mutants HA-L501fs533, causing the disease as already described (Robitaille et al., 2002), HA-FZD4 $\Delta$ VV, which is identical to the wild-type but lacking only the two C-terminal valines and HA-

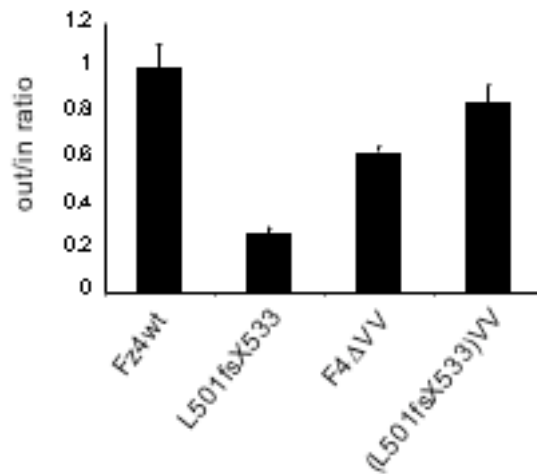
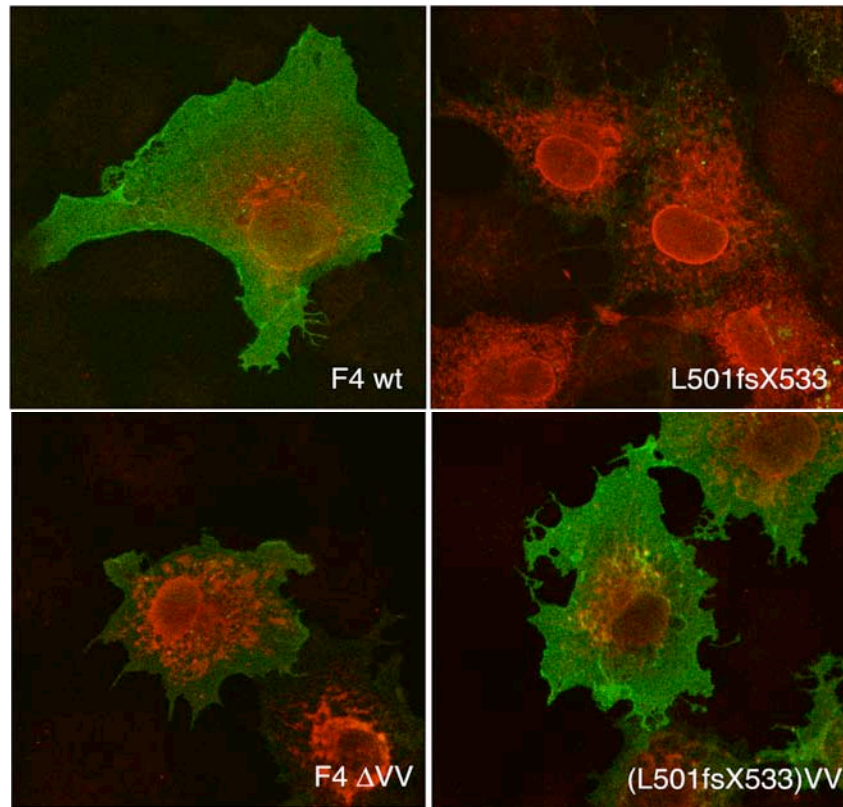
(L501fs533)VV, which is identical to the mutant L501fs533, but bearing two C-terminal valines (fig. 12 ).

Cos7 cells were transiently transfected with these recombinant constructs and after 48 hours the cells were processed for an immunofluorescence-based experiment. Cos7 cells transiently expressing FZD4 constructs were stained with a monoclonal anti-HA antibody before permeabilization and with a polyclonal anti-HA antibody after permeabilization. After this, an anti-IgG mouse fluoresceinated and an anti-IgG rabbit rodaminated were used. The cells were then analyzed under a fluorescence microscopy. As shown in figure 13 (upper panel) as expected, HA-FZD4wt localized primarily in plasma membrane whereas HA-L501fs533 accumulated in the ER and did not show any surface staining. Interestingly, the mutant HA-FZD4 $\Delta$ VV showed an apparent staining of ER but a typical plasma membrane pattern was also clearly visible. Lastly, HA-(L501fs533)VV showed a localization which was completely different from that of HA-L501fs533 as it presented a strong plasma membrane staining which was more similar to that of the HA-FZD4wt one.

To have a quantitative idea of the localization differences between the FZD4 mutants, we calculated the extracellular/intracellular staining ratio for all the constructs. The results were reported in a graph (fig. 13, lower panel) whereby the mutant bearing C-terminal valine accumulates in the plasma membrane in a similar way to FZD4wt, whereas in the absence of valine the protein reaches the cell surface less efficiently and accumulates in the ER. This data clearly indicates that the C-terminal valine is required for an efficient transport from the ER to the plasma membrane of the FZD4 receptor.



**Figure 12. Schematic representations of the HA-Frizzled4 constructs.** Using the HA-Fz4wt as a template the following mutants were generated : HA-L501fs533, causing FEVR, HA-Fz4 $\Delta$ VV, identical to the wild-type but lacking only the two C-terminal valines and HA- (L501fs533)VV, identical to the FEVR-mutant L501fs533, but bearing two C-terminal valines.

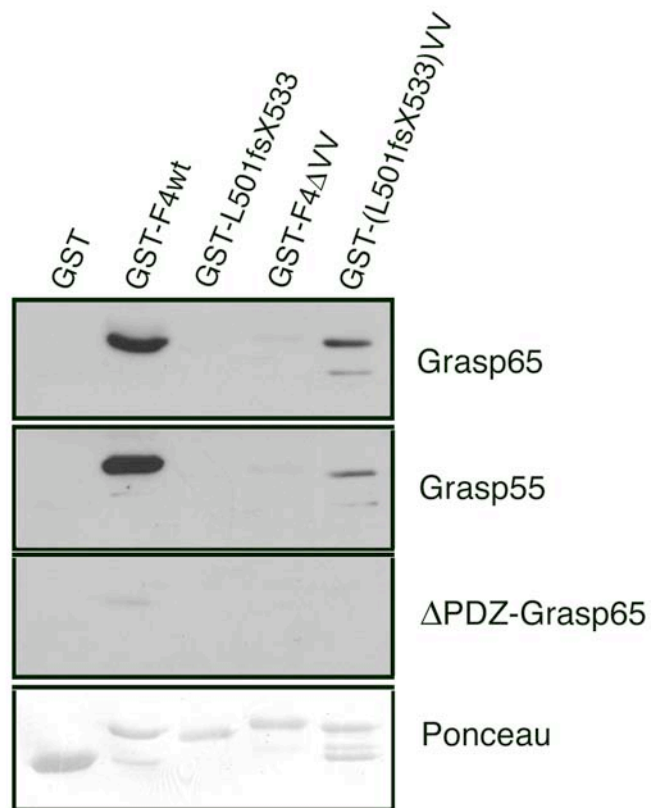


**Figure 13. The C-terminal valine signal enhances intracellular transport of the Frizzled4 receptor.** Cos-7 cells transiently expressing HA-Frizzled4 constructs were stained with a monoclonal anti-HA before the permeabilization (green) and with a polyclonal anti-HA after the permeabilization (red). The ratios extracellular staining/intracellular staining for the constructs were given in the graph.

### 3.2.2 Frizzled4 interacts in vitro with GRASP65 and GRASP55 in a C-terminal-valine-fashion

C-terminal valine in some members of the Frizzled family, like in other proteins bearing this signal, is part of a PDZ domain that interacts with PDZ proteins. In particular, GOPC has been demonstrated to interact with Frizzled4 and to have a role in the translocation of Frizzled proteins to the cell membrane. Moreover, since the previous data obtained for CD8 $\alpha$  demonstrated a direct C-terminal valine dependent interaction between the cytosolic tail of CD8 $\alpha$  and GRASP65 and GRASP55, and showed the role of the two golgins in the transport to the plasma membrane, we decided to test if GRASP65 and GRASP55 play a similar role in the transport of FZD4 to the cell surface. Firstly, we decided to test to see if FZD4wt was able to interact with GRASP65 and GRASP55 and if, eventually, the interaction was mediated by the C-terminal valine of the cytosolic tail of the FZD4 receptor. To do so we performed a Far Western blotting. We generated four recombinant constructs by fusing the cytosolic tails of FZD4wt and the FZD4 mutants described above to the C-terminal of a GST protein. The constructs were transformed in bacteria, afterwards the proteins GST as control, GST-FZD4, GST-L501fs533, GST-FZD4 $\Delta$ VV and GST-(L501fs533)VV were isolated from the bacterial lysates and purified on sepharose-glutathione. They were then analyzed by SDS-PAGE and transferred onto nitrocellulose filters. The filters were overlaid with purified 6xHis-tagged GRASP65, GRASP55,  $\Delta$ PDZ-GRASP65. The bound proteins were detected with an anti-His antibody and ECL. As shown in fig. 14, the cytosolic tail of FZD4 wt bound to both GRASP65 and to GRASP55 whereas the tails of the mutants L501fs533 and FZD4 $\Delta$ VV, which were both lacking C-terminal valine, did not. Interestingly, when we added the C-terminal valine to the tail of the

mutant, L501fs533 recovered the binding to the golgins. Moreover, none of the recombinant proteins interacted with the GRASP65 mutant which lacked the PDZ domains. This data shows a specific interaction between the cytosolic tail of FZD4, indicating that the interaction is C-terminal valine dependent and suggesting that it occurs via PDZ domains which is similar to that observed for CD8 $\alpha$ .



**Figure 14. Frizzled4 interacts directly with GRASP65 and GRASP55 in a C-terminal-valine-fashion.** Purified proteins GST, GST-FZD4, GST-L501fsX533, GST-FZD4 $\Delta$ VV and GST-(L501fsX533)VV were resolved on 12% electrophoresis gel and transferred onto nitrocellulose filter. The filters were incubated with purified proteins His-tagged indicated on the right. The bound proteins were detected with an anti-His antibody.

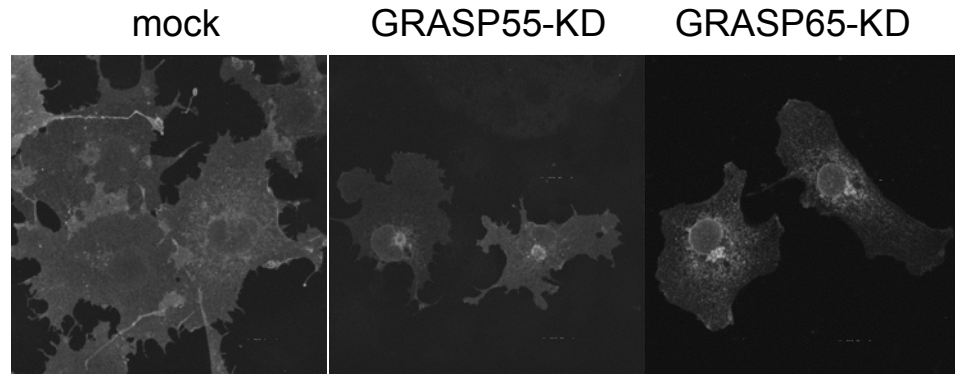
### 3.2.3 GRASP65 and GRASP55 are required for full transport of the receptor FZD4 to the plasma membrane

We have shown that GRASP65 and GRASP55 are required for the efficient transport of CD8 $\alpha$  to the cell surface. Since FZD4 was able to interact with GRASP65 and GRASP55 *in vitro*, we wanted to know if this interaction had a functional significance and, in particular, if the two golgins had a role in the transport of the receptor to the plasma membrane. Therefore, the expression of GRASP65 and GRASP55 in Cos-7 cells was knocked down by RNA interference (RNAi) and the cells subjected to RNAi were transfected for the expression of HA-FZD4wt. After 48 hours, the cells were treated for immunofluorescence as above (par 3.2.1) and analyzed by confocal microscopy (fig.15a). Both the GRASP65 knocked down cells and the GRASP55 knocked down cells showed a weaker plasma membrane staining when compared with the plasma membrane staining of the mock transfected cells. Moreover, in the GRASP65 knocked down cells, FZD4wt seemed to clearly stain the nuclear envelope suggesting a reticular localization and, in both the GRASP65 knocked down cells and the GRASP55 knocked down cells, FZD4wt accumulated in a concentrated perinuclear region which presumably was the Golgi complex. The extracellular staining/intracellular staining ratio provided in the graph (fig. 15b) confirms the features observed: the amount of FZD4 expressed on the cell surface is reduced in the knocked down cells for the golgins.

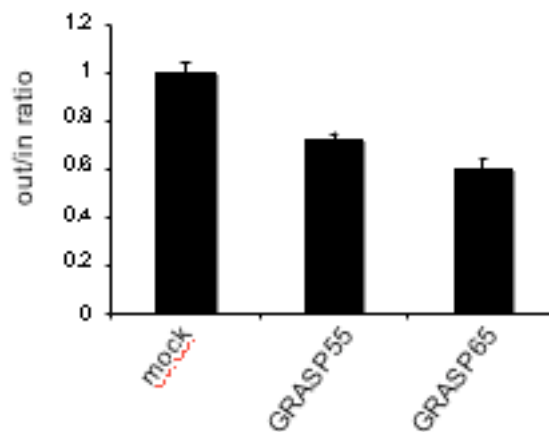
This data suggests that GRASP65 and GRASP55 are needed for the full transport of the FZD4 receptor as they are needed for the transport of CD8 $\alpha$ .



**a**



**b**



**Figure 15. GRASP65 and GRASP55 are required for full transport of the receptor FZD4 to the plasma membrane. a)** The expression of GRASP65 and GRASP55 in Cos-7 cells was knocked down by RNA interference. The cells were then transfected for the expression of HA-FDZ4 wt, treated for immunofluorescence with an antibody anti-HA and analyzed by confocal microscopy. **b)** Cos-7 cells knocked down for the expression of GRASP65 and GRASP55 and transiently expressing HA-FDZ4 were stained with a monoclonal anti-HA before permeabilization and with a polyclonal anti-HA after permeabilization. The extracellular staining/intracellular staining ratio for the constructs were given in the graph.

### **3.3 Conclusions**

In this phase of the project, by mutagenizing the tail of the receptor Frizzled4, by an *in vitro* interaction assay and by the RNA interference technique, we were able to demonstrate that:

- 1) the C-terminal valine enhances the transport of Frizzled4 from the ER to the plasma membrane;
- 2) Frizzled4 interacts *in vitro* with GRASP65 and GRASP55 in a C-terminal valine-dependent fashion;
- 3) GRASP65 and GRASP55 are required for the full transport of the receptor Frizzled4 to the plasma membrane.

## CHAPTER IV

### 4. Materials and methods

#### 4.1 *Materials*

All culture reagents were obtained by Sigma-Aldrich (Milano, Italy). The solid chemical and liquid reagents were obtained from E. Merck (Darmstadt, Germany), Farmitalia Carlo Erba (Milan, Italy), Serva Feinbiochemica (Heidelberg, Germany), BDH (Poole, United Kingdom) and Delchimica (Naples, Italy). All the radiochemicals were obtained from Perkin Elmer (Bruxelles, Belgium). The Protein A-Sepharose CL-4B and the ECL reagents were from Amersham Biosciences (Milan, Italy).

The following antibodies were used: an ascite  $\alpha$ -lumenal domain of VSVG (a kind gift of J. Grunberg); a rabbit anti-Sec31 (Marra et al., 2001); a rabbit anti-HA (Santacruz); a mouse anti-HA (Santacruz), a mouse anti-His (Sigma-Aldrich); a rabbit anti-GRASP65 (Marra et al., 2001). Peroxidase-conjugated anti-mouse and anti-rabbit IgG were from Sigma-Aldrich (Milan, Italy); Texas-Red-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

#### 4.2 *Cell culture and transfections*

Cos-7 cells were cultured in DMEM 10% FCS at 37° C with 5% CO<sub>2</sub>. The cells were transfected using Fugene (Roche) according to the manufacturers' protocols.

### 4.3 Plasmids

To obtain the construct VSV-G-CD8 $\alpha$ , the region encoding for the ectodomain and the transmembrane domain of VSV-G-ts045 were isolated for PCR from the cDNA of the protein by using the following oligos containing BamHI/EcoRI flanking restriction sites:

oligoVSVGBamHIup:

5'-CGCGGATCCATGAAGTGCCTTTTGTACTTAG-3';

oligo VSVGEcoRI down:

5'-CCGGAATTTCGAGAACCAAGAATAGTCCAATG-3';

the PCR product was subcloned in pcDNA3.1 (Invitrogen). The cytosolic tail of CD8 $\alpha$  was isolated by PCR from the cDNA of the human glycoprotein CD8 $\alpha$  by using the following oligos having EcoRI/XhoI flanking restriction sites:

oligo CD8Ecoup:

5'-CCGGAATTCCACAGGAACCGAAGACGT-3';

oligoCD8Xh down:

5'-CCGCTCGAGTTAGACGTATCTCGCCGAAAG-3'.

The PCR product was subcloned downstream of the ectodomain/transmembrane coding region of VSV-Gts045 in pcDNA3.1. To obtain the construct VSV-G-CD8 $\Delta$ YV the following oligos BamHI/XbaI flanking restriction sites and a STOP codon introducing were used: oligoVSVGBamHIup (above described);

oligo XbaVSVG $\Delta$ YV:

5'-GCTCTAGACTATCTCGCCGAAAGGCTGGG-3'.

The PCR product was subcloned in pcDNA3.1.

The construct pCDNA5-HA-FZD4 was kindly provided by M. MacDonald and M.R. Hayden. The mutants L501fsX533, FZD4 $\Delta$ VV and

(L501fsX533)VV were obtained by site direct mutagenesis using the following oligos:

oligoF4delCTup:

5'-TGGTCTGCCAAAACCTTCACACGTGGCAGAAG-3';

oligoF4delCTdw:

5'-AGTTTTGGCAGACCAAATCCACATG-3';

oligoF4insSTOPup:

5'-AAAGGCAGTGAGACTTGAGTGGTATAAGGCTAG-3';

oligoF4insSTOPdw:

5'-AGTCTCACTGCCTTTTCCAGGCTTC-3';

oligo F4addVVup:

5'CCTGGAAAAGGCAGGTGGTATGAGACTGTGGTATAAGG-3';

oligo F4addVVdw:

5'-CTGCCTTTTCCAGGCTTCA CCCAA-3'.

In order to obtain the construct GST-FZD4, the sequence encoding the cytosolic tail of FZD4 was obtained by PCR using pcDNA5-HA-FZD4 as a template and the following oligos with BamHI/XhoI flanking restriction sites:

oligoFZD4tail up:

5'-CAGGATCCACTCTTCACACGTGGCA-3';

oligoFZD4tail down:

5'-CACTCGAGGGCAACTAGAAGGCACAG-3';

The PCR product encoding the cytosolic tail of FZD4 was subcloned downstream the region encoding GST in a pGEX-4T-1 vector (GE Healthcare)

The mutants GST-(L501fsX533), GST-FZD4 $\Delta$ VV and GST-(L501fsX533)VV were obtained by site direct mutagenesis using the following oligos:

oligoGST-FZD4 delCT up:

5'-GTTCCGCGTGGATCCACTTCACACGTGGCAGAAG-3'

oligoGST-FZD4 delCT down:

5'-TGGATCCACGCGGAACCAGATCCGATTTTG-3'

oligoGST-FZD4 insSTOP up:

5'-AAAGGCAGTGAGACTTGAGTGGTATAAGAATTC-3'

oligoF4insSTOPdw; oligo F4addVVup; oligo F4addVVdw (above described).

#### ***4.4 Anterograde transport analysis of the VSV-G-based chimerae***

Actively growing Cos-7 cells were transfected with the expression vectors encoding the chimerae of the temperature-sensitive mutant VSV-G-ts045, GFP-VSVG, VSV-G-CD8 $\alpha$  and VSV-G-CD8 $\Delta$ YV by using Fugene 6.0, in accordance with the manufacturers' instructions (Roche) and kept at 39°C to retain the chimeric protein in the ER. 48 hours after transfection, cells were moved to the permissive temperature (32°C), to 15°C or 10°C (for the temperature block experiment) and the cells were analyzed by fluorescence analysis as indicated.

#### ***4.5 In vitro budding assay***

Monolayers of stably transfected FRT cells were pulse-labeled for 15 minutes with a mix of <sup>35</sup>S-Cys and <sup>35</sup>S-Met and were scraped in an ice-cold buffer B (10  $\mu$ g/ml leupeptin, 5  $\mu$ g/ml pepstatin A, 2  $\mu$ g/ml aprotinin, 25  $\mu$ g/ml ALLN in PBS). The cell pellet was re-suspended in 0.4 ml of buffer F (10mM Hepes-KOH pH 7.2, 250 mM sorbitol, 10 mM KOAc, 1.5 mM Mg(OAc)<sub>2</sub>, plus protease inhibitors), and then passed through a 22-gauge needle 20 times and centrifuged at 1,6x10<sup>4</sup> g for 3 minutes at 4°C in an

Eppendorf centrifuge in order to obtain microsomal membranes. The pellet was then re-suspended in 80  $\mu$ l of Buffer E (50 mM Hepes-KOH pH 7.2, 250mM sorbitol, 70 mM KOAc, 2.5 mM Mg(OAc)<sub>2</sub>, 5 mM potassium EGTA, plus protease inhibitors). The complete incubation mixtures containing 50  $\mu$ g microsomes, 600  $\mu$ g of rat cytosol, 1.5 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, and 4 U/ml creatine kinase in a final volume of 80  $\mu$ l of Buffer E were incubated either at 37°C or placed on ice for 20 minutes. Reactions were terminated by transferring the tubes to ice, followed by centrifugation for 3 minutes at  $1.6 \times 10^4$  g at 4 °C to obtain a medium speed pellet (M) and a supernatant fraction. The supernatant was centrifuged for 10 minutes at  $10 \times 10^4$  g at 4 °C in a Beckam Coulter TL-100 centrifuge to obtain a high-speed pellet (V). Subsequently, both the M (representing the parental membrane) and V (representing the budded membranes) fractions were subjected to lysis and immunoprecipitation using an anti-CD8 antibody.

#### ***4.6 Indirect immunofluorescence***

Cos-7 cells transiently transfected as described above were grown on glass coverslips fixed in PBS 4% paraformaldehyde for 10 minutes. They were then permeabilized with 0,05 % saponin in blocking buffer (0.5% BSA, 50 mM ClNH<sub>4</sub>, 0.02 NaN<sub>3</sub> in PBS) for 30 minutes, washed in PBS and incubated with the primary antibodies diluted in blocking buffer for 1 hour and then with the appropriate secondary antibodies diluted in blocking buffer for 1 hour at room temperature. For the immunofluorescence experiments of Frizzled-4 mutants localization, Cos-7 cells transiently transfected, grown on glass coverslips, fixed in PBS 4% paraformaldehyde for 10 minutes, were blocked in blocking buffer for 30 minutes, incubated with a polyclonal anti-HA diluted in blocking buffer, fixed again in PBS

4% paraformaldehyde for 10 minutes, permeabilized in 0,05 % saponin blocking buffer for 30 minutes and then incubated with a monoclonal anti-HA for 1 hour. The cells were then incubated with the appropriate FITC or Texas Red conjugated secondary antibodies for 1 hour. Afterwards the coverslips were mounted on slides with Mowiol and were analyzed with an LSM 510 confocal laser scanning microscope.

#### ***4.7 Far western Blotting***

GST proteins were produced: the plasmids were transformed into BL-21 bacteria. The bacteria were lysed with extraction buffer (Hepes 20 mM pH 7.4, MgCl<sub>2</sub> 2mM, KCl 50 mM, NP40 0.5%, glycerol 20%, EDTA 2mM) and then subjected to sonication. Bacteria cell debris were pelleted under centrifugation for 30 minutes at 10000 rpm, 4°C. The lysates were incubated with Glutathione sepharose 4B (Amersham) at 4° for 2 hours with gentle agitation. The glutathione sepharose pellets were washed 4 times with 5 bed volumes of extraction buffer. The conjugated proteins were quantified using a spectrophotometer.

The GST proteins (5 µg) were resolved on linear 12.5% polyacrilamide gels and then blotted onto nitrocellulose filters. The filters were stained with Ponceau, washed with 5% acetic acid and incubated overnight in blocking buffer (4% dry non fat milk, 0.2% Triton X100, 0.02% NaN<sub>3</sub> in PBS). They were then incubated with purified His tagged proteins (kindly provided by lab. De Matteis, Consorzio Mario Negri Sud) at a concentration of 1µM in blocking buffer for 8 hours at 4°. After extensive washing with PBS 0.1% Tween20, the filters were incubated with a monoclonal anti-His and then with a peroxidase-conjugated secondary antibody. After washing, the bound antibodies were detected by ECL.



#### ***4.8 RNA interference***

The siRNAs used were of human GRASP65 (NM\_031899) and human GRASP55 (NM\_015530) consisting of a mixture of four siRNA duplexes that were selected using the Dharmacon SMART selection process and SMART pool algorithm. They were obtained from Dharmacon (Lafayette, CO, USA). The COS-7 cells were plated at 30% confluence in 24-well plates and transfected with 50 pmol of GRASP65 or GRASP55 siRNA using Oligofectamine (Invitrogen, Milan, Italy) according to the manufacturer's protocol. 48 hours after the initial siRNA treatment, the cells were transfected with VSV-G-based or HA-FZD4 constructs. Cells transfected with VSV-G-based constructs were kept for 16 hours at 40 °C before the transport assay was performed. Then the cells were processed for indirect immunofluorescence.

## Discussion

In the present study, we have shown that the C-terminal valine has a role in transport to the cell surface of the glycoprotein CD8 $\alpha$  and the receptor Frizzled4. We have shown that it functions in a very early step of the secretory pathway. It has been well established, at least for CD8 $\alpha$  but just as likely for FZD4 transport as well, that the C-terminal valine does not play a role in the exit from the ER. We have also demonstrated that GRASP65 and GRASP55 decode the information provided by the C-terminal valine motif and sequentially promote the anterograde transport of CD8 $\alpha$  and FZD4 along the secretory pathway. The GRASPs knocking down by RNAi or the C-terminal valine's removal results in the same transport defect and mutations in the motif which result in the altered trafficking lead to the loss of direct binding of CD8 $\alpha$  and FZD4 with the GRASPs. Surprisingly, the GRASPs do not directly contribute to the transport of membrane cargo proteins not bearing the C-terminal valine motif and therefore this mechanism is specific for a subset of membrane proteins. Therefore, our data provides an explanation for the molecular mechanisms behind the previously reported role of the C-terminal valine motifs in anterograde transport (Boyle et al., 2006; Crambert et al., 2004; Iodice et al., 2001; Paulhe et al., 2004; Urena et al., 1999) and for the GRASPs binding to specific cargoes (Barr et al., 2001; Kuo et al., 2000). Our data also clearly establishes that as well as both GRASP65 and GRASP55 having their functions in Golgi architecture, in mitotic progression and in unconventional protein secretion, they also have direct roles in the 'conventional' transport of secretory cargo between the ER, the IC and the Golgi complex. Finally, our data opens up new and interesting scenarios for the regulation of the surface expression of receptors such as

CD8 and FZD4, which strongly suggests that an alteration in this mechanism is the cause of a dominant form of human FEVR.

What might be the distinctive properties of the C-terminal-valine-bearing cargo proteins that make them sensitive to the GRASPs? Different possibilities can be envisioned here. These proteins might have an as-yet-undefined ‘retrograde’ signal that would usually be overridden by the GRASP-interacting anterograde C-terminal valine motif. In the absence of either the valine signal or the GRASPs, the retrograde motif would mediate the recycling of these cargo proteins to the ER, as has been suggested for the NMDA receptor, where a C-terminal valine motif counteracts the activity of an ER-retention arginine-based motif in the protein (Standley et al., 2000; Wenthold et al., 2003). As an additional possible mechanism, the C-terminal valine could promote an active sorting of these GRASP-sensitive cargo proteins into anterogradely moving carriers, thereby accelerating their transport to the Golgi complex. In the absence of the valine or of the GRASPs, these cargoes would instead be transported via a bulk-flow mechanism which would necessarily be less effective than the mechanism based on active sorting. In the hypothesis that we believe is more consistent with the available evidence and results reported here, GRASP65 and GRASP55 are envisaged as having specific functions at two different stages that are temporally and spatially distinct. The GRASP65-sensitive step corresponds to an early step in ER-to-IC transport although we can exclude the possibility that this valine–GRASPs interaction has a role in promoting COPII recruitment to the ERES and/or in the budding of carriers from the ER.

A similar conclusion has recently been reported for yeast where, despite the ability of the GRASPs homologue Grh1 to bind to COPII, it is not required for the budding of COPII vesicles (Behnia et al., 2007). Therefore, the action site of this valine–GRASP65 interaction system has to be placed

at a post-ER and pre-Golgi station, i.e. the IC. Unfortunately too little is, at present, known at the molecular level about cargo protein's entry into and transit through the IC, to define in detail the molecular mechanisms promoting the anterograde transport of these C-terminal-valine-bearing proteins. What has so far been established is the compositional heterogeneity of the IC with its 'early' elements that are physically close to, but distinct from, the ERES and its 'late' elements that are closer to the Golgi complex (Marra et al., 2001). In this context, active sorting of a cargo protein from the early to the late IC components (through its interaction with GRASP65) would offer a kinetic transport advantage. This would occur through the recruitment of the GRASP65–GM130 machinery which can form a complex with the transiting C-terminal-valine-bearing cargo, and which has a recognized role in promoting the incorporation of ER-derived carriers into the Golgi complex (Marra et al., 2007). The GRASP55-sensitive transport step for these GRASP-sensitive cargoes appears to be within the Golgi complex and it is presumably related to the *cis*-to-medial Golgi transition of the cargoes, considering the block of VSVG–CD8 $\alpha$  in the *cis*/medial Golgi compartment in the condition of knocked down levels of GRASP55 and considering the medial Golgi localisation of GRASP55. The molecular mechanisms involved in this transport block have yet to be defined. An intriguing possibility is that in the absence of GRASP55, the C-terminal-valine-bearing cargo proteins remain bound to GRASP65, and are therefore stacked in early Golgi compartments.

Finally, interactions with the GRASPs are likely to have an important role in the physiology of CD8 and Fz4, two plasma-membrane receptors. CD8 is a glycoprotein complex that is mainly expressed in cytotoxic T lymphocytes. It consists of two subunits that can associate as homodimers and heterodimers: the  $\alpha$  subunit, which has a C-terminal valine; and the  $\beta$

subunit, which is devoid of this signal (Parnes, 1989). CD8 $\alpha\beta$  is the main functional co-receptor, although CD8 $\alpha\alpha$  is also expressed at the cell surface and is functional, whereas CD8 $\beta\beta$  is inactive and is retained in the ER (Devine et al., 2000; Dialynas et al., 1981; DiSanto et al., 1988; Hennecke and Cosson, 1993; Norment and Littman, 1988). The co-expression of CD8 $\alpha$  relocalises CD8 $\beta$  to the cell surface (Hennecke and Cosson, 1993, Schmidt-Ullrich and Eichmann, 1990). These considerations thus highlight the driving role that the valine signal plays in the promotion of the exposure of these receptors at the plasma membrane and hence their function. FZD4 and the other members of the FZD family of Wnt receptors also form homo-oligomers and hetero-oligomers (Kaykas et al., 2004). Recently, it has been shown that this oligomerisation occurs in the ER (Kaykas et al., 2004), and that the FZD4 mutation responsible for FEVR does not allow anterograde transport of the mutated proteins and blocks the transport of the wild-type FZD chains upon oligomerization (Kaykas et al., 2004). This mechanism would explain the dominant effects of the FZD4-FEVR mutant in heterozygous FEVR patients (Robitaille et al., 2002). The absence of FZD4 receptor expression at the plasma membrane results in a signalling defect during embryogenesis that leads to defective angiogenesis, aberrant neo vascularization and exudative retinopathy (Robitaille et al., 2002). Our results can now provide a molecular explanation for the intracellular retention of FZD4-FEVR, as the inability of the mutant protein to interact with GRASP65 and GRASP55.

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